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Immune responses of cattle to antigens of Pasteurella multocida

Ronald W. Griffith
Iowa State University

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by

Ronald W. Griffith

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INTRODUCTION

Pasteurella multocida is known to be a major etiologic factor in bovine respiratory disease. Respiratory diseases in the United States cause millions of dollars in losses annually in the cattle industry. Such losses include not only deaths but also excessive shrinkage, cost of treatment, inefficient feed conversion, and delayed marketing. In a recent study, Jensen et al. (1976) surveyed a large population of feedlot cattle. Approximately 5.1% of the cattle became ill and 18.9% of those died. Approximately three quarters of the illness was attributed to respiratory disease. Pasteurella species alone or in combination with other agents were recovered from 62 percent of the lungs at necropsy. Although no good statistics are available, a significant percentage of respiratory tract infections involve Pasteurella multocida as either a primary or secondary disease producing agent.

The recognition of the role of P. multocida in bovine respiratory disease has led to the development and use of various bacterin products. The use of most of these products has proved disappointing for the following reasons: 1) Type A P. multocida is poorly antigenic. At least two doses and often more are required to give a significant level of protection. The cost and inconvenience of multiple dosage regimens has not been well-received by the cattle industry in this country. 2) Bacterins probably do not initiate a

traditional cellular type response. Such a response may be an important component of the local pulmonary defense against P. multocida. 3) P. multocida bacterins are most effective when immunization is performed prior to shipment of cattle to feedlots. Immunization less than two weeks prior to, during, or after shipment is relatively ineffective.

In addition to the above problems, the efficacy of bacterins as prophylactic agents is questionable. The antibody response to these agents is of a low order. It appears that stress factors associated with shipping and infection of the respiratory tract with certain virus agents are capable of negating the protective immune response to killed bacterins. In addition, immunosuppression and toxicity due to certain components of killed bacterins may be a problem. Friend et al. (1977) demonstrated that cattle immunized with a P. hemolytica bacterin had a higher incidence and greater severity of respiratory disease caused by this agent than control animals.

Little definitive investigation has been carried out as to the mechanisms by which cattle respond to P. multocida Type A infection. Other species of animals have been utilized as models but the majority of those investigations were concerned with systemic and not pneumonic mechanisms of resistance and clearance. There is a relative dearth of information on the immune response of cattle to this agent.

Recent work by Maheswaran and Thies (1979) has indicated that,

in addition to a humoral immune response, cattle may mount a cell-mediated response. The goals of this investigation have been to: 1) Isolate and characterize various immunogenic fractions from a Type A P. multocida and 2) Characterize the nature of the immune response of the bovine to these immunogenic fractions.

LITERATURE REVIEW

History

Diseases now known to be caused by Pasteurella species were reported long before the isolation of the etiological agent. These organisms were thought to play a role in large epornitics among fowl in Europe in the latter half of the eighteenth century (Brogden, 1977). Maillet, in 1836, was the first to use the name fowl cholera in connection with these epornitics (Manninger, 1929).

Dadd (1859) discussed the symptoms and treatment of respiratory diseases called catarrh or Hoose and enzootic catarrh in cattle. He stated that "catarrh is nothing more than a common cold, induced by the ordinary causes such as exposure and errors in diet and management." He inferred a hereditary predisposition and stated that, when an animal is removed from a warm to a colder region, it is apt to contract catarrh.

Renault and Delafond presented the first experimental evidence for the transmissibility of fowl cholera around the middle of the nineteenth century (Manninger, 1929). Rivolta in 1877 and Perroncito in 1878 described the presence of microbes having a rounded form appearing singly or in pairs in the blood of chickens infected with fowl cholera (Harshfield, 1965). Bollinger, in 1878, made what was probably the first significant report of what was to be labeled pasteurellosis (Carter, 1967a). He investigated an outbreak of fatal disease among wild animals and cattle. Toussaint (1879) was the first to isolate and describe P. multocida from the blood of a chicken. The most complete description of the organism was made by Pasteur (1880a, b).

Kitt, in 1885, made a comparative study of the organisms producing fowl cholera, rabbit septicemia, swine septicemia, and septicemia of wild animals and cattle. Because of the similarities between the causative organisms, he referred to them as Bacterium bipolare multocidum (Rosenbusch, 1937). In 1886, Hueppe noted the similarities of the diseases caused by these organisms and suggested the name septicemia hemorrhagica for the disease (Rosenbusch and Merchant, 1939).

In 1886, Poels was the first to describe the organism of calf pleuropneumonia (Carter, 1967a). The strain present in buffalo was reported by Oreste and Armanni in 1887 (Carter, 1967a). In 1895, Moore reported finding members of the hemorrhagic septicemia group present on the mucous membranes of the respiratory tracts of apparently normal cattle, sheep, swine, dogs, and cats (Hagan and Bruner, 1957).

Trevisan in 1887 suggested the genus name Pasteurella for this group in honor of Louis Pasteur (Gray, 1913). He listed three species for the genus: Pasteurella davainei, Pasteurella choleragallinarum, and Pasteurella suis. There was an early tendency to use zoological classifications based on the host species infected. Thus, in 1896, Flügge referred to the various Pasteurella organisms as Bacillus bovisepticus, B. suisepticus, etc. Lignières (1900) listed the organisms causing pasteurellosis as Pasteurella aviaire, P. bovine, P. ovine, P. porcine, etc. Lignières' nomenclature system was widely accepted by bacteriologists and the genus Pasteurella became well-established. However, the species names suggested by Flügge were adopted; hence

Pasteurella aviseptica, -boviseptica, -oviseptica, -suiseptica, -equiseptica, -leptiseptica, and -muriseptica (Carter, 1967a). However, it became increasingly apparent that these organisms were morphologically, biochemically, serologically, and pathologically similar. An attempt was made to include all these strains under one name. Wilson and Miles in 1929 suggested that these Pasteurellas be referred to as P. septica (Buchanan et al., 1966). Rosenbusch and Merchant (1939) proposed the name P. multocida because the first inclusive name, Bacterium bipolare multocidum, was first used by Kitt in 1885. Pasteurella multocida is the name universally accepted at this time.

It was recognized early that an atypical group of Pasteurella organisms was also involved in disease in cattle and sheep. In 1921, Jones reported a separate group of Pasteurella organisms which fermented lactose, did not produce indol and which hemolyzed blood. In 1932, Newsom and Cross studied this group in detail. They reported this group to be identical to Jones' Group I and placed the atypical strains in a separate species which they called Pasteurella hemolytica. This name has also been universally accepted.

Physical characteristics of P. multocida

The usual form of Pasteurella multocida is that of a short, rounded, small cocobacillary rod. Cultures can be quite pleomorphic, however, especially if grown under unfavorable conditions. Old laboratory cultures will often contain long filamentous forms. The organisms vary in size from 0.25 to 0.4 μm by 0.6 to 2.6 μm.

As viewed by electron microscopy, the organism has a structure similar in many respects to other gram negative bacteria (Glauert and Thornley, 1969; Brogden, 1977). The cytoplasm of the cell is delineated by a plasma membrane. Peripheral to this is an outer unit membrane which presents a contoured rugated surface topography. Brogden described a dense intermediate layer and an electron transparent interspace between these two membranes. Negatively stained preparations of both broth and agar cultivated cultures revealed the presence of microfilaments on the surface of two strains of P. multocida. These filaments were 400-500 Å in length and 15-30 Å in diameter. These structures were not observed on thin sectioned specimens.

P. multocida shows the surface blebs observed with other gram negative organisms (Glauert and Thornley, 1969; Devoe and Gilchrist, 1973). These blebs or finger-like projections are thought to be evaginations of the outer cell membrane and consist of the same trilaminar arrangement (Brogden, 1977). Devoe and Gilchrist (1973) worked with Neisseria meningitidis and found that 18% of the endotoxin produced by the organism was present in such blebs found free in the supernatant fluids.

Many isolates of Pasteurella multocida also possess a capsule. The amount and type of capsule tends to vary greatly from isolate to isolate. The amount of capsule also appears to be a function of the serotype to which a particular isolate belongs. Carter's Type A possess the largest amount of capsular material (Carter and Rundell, 1975). To a large extent the capsule of Type A strains of P. multocida

is composed of hyaluronic acid (Carter and Annau, 1953; Bain, 1954). The hyaluronic acid would appear to be in the form of a framework rather than in a discrete layer (Carter, 1967b).

The capsules associated with some of the Type D organisms contain much less hyaluronic acid (Carter and Subronto, 1973). The capsules of Types B and E contain a greater amount of protein material than do either Types A or D (Penn and Nagy, 1974). Not all isolates possess capsules. Bain (1957) reported that none of 20 canine and feline isolates was capsulated. Smith (1958) surveyed 103 isolates and found that the majority of the canine and feline isolates were noncapsulated. Twenty of 28 porcine cultures and three of five bovine cultures were capsulated, however. There is a tendency for isolates to produce less capsular material or lose it altogether (dissociate) on repeated subculture in broth medium (Elberg and Ho, 1950). This dissociation has given rise to various designations for the same variant. Considerable confusion is evident in the literature. Webster and Burn (1926) applied the term mucoid to those isolates having a great amount of capsular material. This term is still in common use. DeKruif (1921) used the terms diffuse and granular to describe the colony types. Hughes (1930), observing the appearance of the colony under obliquely transmitted light, classified the dissociants as either fluorescent, intermediate or blue. Anderson et al. (1929) used the term rough. This term has been retained in the literature. Braun (1953) suggested the terms mucoid, smooth and rough be employed to designate the principal colonial variants of bacteria. This has gained wide acceptance. Carter (1957a) suggested

that the term "antigenically rough" be employed for those smooth cultures which fell into DeKruif's granular or Hughes' blue categories. Heddleston et al. (1964) used the term "gray" to describe a variant of the blue colony type. Since the term fluorescent used by Hughes (1930) was technically incorrect, it was replaced by the term iridescent. Unfortunately, a combination of these terms is in common use today to describe the dissociants of P. multocida.

Typing Systems

Classification systems for Pasteurella multocida have been reviewed extensively by Cornelius (1929), Carter (1967b), and Brogden (1977). No attempt will be made here to give a detailed discussion of the evolution of the various typing schemes. However, a brief outline of the typing systems employed is germaine. Little and Lyon (1943) described three distinct serological types within the nonhemolytic Pasteurella. They based their classification system on a rapid slide agglutination procedure and a passive serum protection test in mice. Roberts (1947) was able to divide P. multocida into four groups on the basis of serum protection tests in mice. These groups were designated I, II, III, and IV. Carter (1955, 1957b, 1959, and 1963) divided his isolates into four capsular types on the basis of the indirect hemagglutination test. These were designated Types A, B, D, and E. A fifth group (C), proposed earlier, was dropped because of difficulties in recognition (Carter 1959, 1963).

A nonserological procedure for identifying Type A isolates was introduced by Carter and Rundell (1975). They employed staphylococcal hyaluronidase to inhibit the production of capsules by Type A cultures. This resulted in a marked diminution in size of the colonies on blood agar. Type B and E isolates do not produce hyaluronic acid; thus, no effect on these types was observed. Some Type D isolates showed a slight reduction in colony size.

Carter and Subronto (1973) employed the acriflavine reaction for identification of Type D isolates of P. multocida. They found that serotype D produced a characteristic coarse flocculation as seen in the slide agglutination procedure. Having used this procedure on over 100 cultures, it was concluded that it could replace the more involved indirect hemagglutination test.

The serologic features of the somatic antigens of P. multocida were a puzzle prior to the studies of Namioka and associates in the 1960's (Namioka and Bruner, 1963; Namioka and Murata, 1961a, b, c; Namioka and Murata, 1964). They employed antigens treated with 1N HCl in a slide agglutination test. They were able to obtain "specific factor" O sera in rabbits by absorbing the sera with the HCl-treated antigen of cross reacting strains. Their cultures fell into ten somatic groups. Combined with the capsular typing system employed by Carter, 15 serotypes were reported by Namioka (1973). Namioka and Murata (1961c) proposed that serotypes be identified by listing first the somatic (O) antigen followed by the specific capsular antigen, e.g., 7:A. The complexity of the somatic antigens was compounded by the demonstration

of a number of O subgroups (Namioka and Murata, 1961c). A strong correlation between epizootiology and the serotyping system outlined above was made by Namioka and Murata (1964). Fowl cholera was shown to be caused by Serotypes 5:A, 8a:A, and 9:A. The causative agent of hemorrhagic septicemia in cattle was found to be 6:B. Namioka and Bruner (1963) reported that cultures belonging to 1:A, 3:A, 1:D, 2:D, and 4:D were associated with pneumonia in cattle and sheep. The procedures employed for determining Namioka's somatic types have not been widely used (Brogden, 1977). Difficulties are encountered in the preparation of "specific factor" sera in addition to problems with cross reactions.

Prince and Smith (1966a) investigated the antigenic structure of P. multocida by using immunoelectrophoresis. Eighteen soluble antigens were identified. Sixteen of these were common to all of 17 strains of avian and bovine origin (Prince and Smith, 1966b). The remaining two antigens, designated alpha and beta, were type specific. The heat-stable beta antigen was used in the gel diffusion precipitin test to type unknown isolates.

Heddleston et al. (1972a) reported on the use of a gel diffusion precipitin test for serotyping avian isolates of P. multocida. This system has been extended (Heddleston et al., 1972b; Blackburn et al., 1975) and is now composed of sixteen serotypes (Brogden, 1977). Numerous investigations dealing with the distribution of serotypes have been reported since the establishment of this typing system (Brogden, 1977).

Carter (1976) proposed that P. multocida be divided into five

biotypes. These biotypes would be identified on the basis of the following characteristics: hyaluronidase decapsulation, acriflavine flocculation, colonial iridescence, fermentation of carbohydrates, mouse pathogenicity and serum protection tests. He proposed a 1) mucoid biotype, 2) hemorrhagic septicemia biotype, 3) porcine biotype, 4) canine biotype, and 5) feline biotype. The mucoid biotype would encompass the Type A organisms. The hemorrhagic septicemia biotype would be analogous to Types B and E. The porcine biotype appears to be the same as the Type D organisms. The canine and feline biotypes would be characterized by their lack of hyaluronic acid, noniridescence and fermentation patterns. The canine biotype would have low mouse pathogenicity and the feline biotype relatively high mouse pathogenicity. This system of biotypes has not been widely adopted.

Antigens

Elucidation of the antigenic makeup of P. multocida has proven difficult. It has been complicated by the fact that major differences are present in the capsules and cell walls of the various serotypes. In addition, the propensity of the organism to dissociate gives rise to quantitative differences in antigenic makeup. In some cases these have been construed to be qualitative in nature (Carter and Annau, 1953).

Boivin and Mesrobeanu (1935) presented a method for extracting relatively undenatured envelope substances from gram negative bacteria by using trichloroacetic acid (TCA). They found that a relatively complete antigen complex was obtained which was soluble, nondialysable,

antigenic and toxic. It precipitated in the cold in the case of some bacteria. It remained toxic in neutral solutions but antigenicity was lost by heating to 100-120° C. In strongly acid medium and with heating it lost its toxicity and antigenicity and was cleaved into a precipitate and a residual "free polysaccharide" which remained in solution. They worked primarily with the Salmonella group but demonstrated similar materials in Proteus, Pseudomonas and other bacteria. Pirosky (1938) reported the recovery of a Boivin type antigen from smooth and rough variants of an avian strain of P. multocida. He found the extracts to be toxic, protective and serologically specific.

TCA-extracted antigens are never immunologically or chemically identical from one batch to another (Staub, 1967). The nitrogen content may vary from 2-4% with a given bacterial species. The purity of this material can be improved with ultracentrifugation or with gel filtration (Staub, 1967). These processes remove free proteins, nucleic acids and degraded polysaccharides which make up approximately 20% of the whole antigen. The TCA extraction results in the most undenatured cell wall material. This process is also the least efficient at removing cell wall material; thus, a large amount of material remains with the cell which can be extracted by other means. This antigenic preparation is best characterized as a lipopolysaccharide-protein complex.

Carter (1952) isolated what he presumed to be a capsular polysaccharide substance from four cultures of P. multocida by using

two different methods. The first method involved washing bacteria from an agar plate with saline and heating them at 56° C for one hour. The organisms were centrifuged out and the supernatant diluted and used in a precipitation test. The second method was adapted from MacPherson (1948). Briefly, this involved treating agar-grown bacteria with 1 M sodium acetate and ethanol followed by phenol and subsequent extraction by ethanol and saline. This antigen was then used in a precipitation test. He determined that there were two principal antigenic components to P. multocida: 1) A type specific, soluble capsular polysaccharide, and 2) a somatic antigen common to all members of the species.

Carter and Annau (1953) described the isolation of capsular polysaccharides from the colonial variants of P. multocida. The polysaccharides were isolated by heating at 56° C for 1 hr. followed by centrifugation to remove bacteria. The supernatant was precipitated with 3 volumes of ethanol and the precipitate resolubilized in distilled water and dried. The polysaccharides from the fluorescent variant were found to be highly protective in mice. Those of the mucoid (M) variants were much less protective. The M polysaccharide was found to be relatively inactive serologically. Both substances were nontoxic for mice in doses up to 3 mg.

Yaw and Kakavas (1957) removed the capsular polysaccharide by Carter's method from a Type 1 (Little and Lyon, 1943) strain of P. multocida. The whole cells remaining after this extraction were found to be agglutinable in rabbit antiserum to formalin-killed cells. The

capsular polysaccharide was found to be protective in mice and chickens; however, the heat-killed washed cells were protective only in chickens. This indicated a species difference in the response to various antigens of P. multocida. Similar work with Yersinia pestis had shown that different components of the bacterium were responsible for immunization in different host species (Shutze, 1932).

A method for extracting lipopolysaccharide materials from bacteria using phenol was first described by Palmer and Gerlough (1940). Working with Salmonella typhosa, they isolated a phenol-insoluble material which was protective in mice and toxic in mice and rabbits. It was shown to give a strong precipitin reaction with anti-typhoid serum produced in the rabbit. Westphal et al. (1952) simplified this procedure by shaking bacteria in an emulsion of phenol and water at 5 to 10° C. The proteins, which were soluble in the phenol phase, separated from the water-soluble nucleic acids, polysaccharides and lipopolysaccharides. The aqueous phase could then be dialyzed against distilled water and the lipopolysaccharide purified by ultracentrifugation or other means (Staub, 1967; Westphal and Jann, 1965).

MacLennan and Rondle (1957) extracted a type specific lipopolysaccharide from Roberts' Types I, III and V using the Westphal extraction. They found the lipopolysaccharides to be poorly water soluble following lyophilization. The materials were pyrogenic in rabbits but not lethal in that species, in mice, or in guinea pigs at a level of 500 µg. In addition to the lipopolysaccharide (P), they observed another heat-stable antigen on gel diffusion precipitation

which they referred to as S. A rough variant of the Type V strain produced the S but not the P antigen, thus indicating the association of the S antigen with the capsule. Analysis of the acid hydrolysate of the lipopolysaccharide revealed the presence of aldoheptose sugars and glucosamine.

Perreau and Petit (1963) isolated a glycolipid endotoxin antigen from Type E. (P. multocida) by Westphal's method. It was found to be identical to the lipopolysaccharide of Type B. However, they found a qualitative difference between the antigen of Types B and E.

Bain (1955) extracted a polysaccharide-protein complex from the capsules of P. multocida Type I (Roberts). The complex was immunogenic in mice and could absorb the protective power from rabbit antisera to whole bacilli.

Knox and Bain (1960) extracted cell pastes of Type I (Roberts) with 2.5% NaCl, followed by isoelectric precipitation of protein at pH 3.8 and precipitation of the polysaccharide with ethanol. They found that the polysaccharide could be fractionated into products containing varying proportions of glucosamine and fructose. The polysaccharide was produced by both iridescent and blue variants; however, the blue variant released the polysaccharide into the surrounding medium almost entirely. The polysaccharide was recovered most abundantly from agar grown cells. In broth cultures the polysaccharide passed readily into solution, rendering cells which were readily agglutinable by specific antiserum. The saline-extracted crude polysaccharide did not induce protective immunity in mice when injected subcutaneously or intraperitoneally in

doses ranging from 2 to 100 µg. Gel diffusion precipitin tests using sera from rabbits hyperimmunized against whole cells reacted with crude polysaccharide to give a variable number of lines. When the crude polysaccharide was trypsinized only two lines usually remained. Trypsinization also abolished the protective power of the polysaccharide when injected subcutaneously into mice, rabbits and cattle. It would then appear that the purified polysaccharides are haptens and depend upon the protein moiety for antigenicity.

Bain and Knox (1961), working primarily with Roberts' Type I, extracted P. multocida first with saline and then followed this with a phenol-water extraction. The lipopolysaccharide obtained by the phenol-water extraction was further purified by ultracentrifugation at 105,000 g for 2 hours. The lipopolysaccharide was found to contain galactose, glucose and glucosamine in addition to the D-glycero-L mannoheptose reported by MacLennan and Rondle (1957); Forester, Davies and Crumpton (1958). The lipopolysaccharide was found to be highly toxic to rabbits in doses of 500 µg I.V. Two-week old calves collapsed when given a dose of 300 to 700 µg but recovered. Low doses elicited a febrile response in rabbits but caused hypothermia in mice. The lipopolysaccharide produced precipitins in rabbits detectable by gel diffusion precipitation; however, sera from these rabbits would not passively protect other rabbits against a 500 µg dose. The purified lipopolysaccharide induced only a 20% protection level in mice against a homologous challenge.

Amies in 1951 demonstrated that potassium thiocyanate (KSCN) removed

the envelope from Yersinia pestis (Amies, 1951). He isolated a protein fraction from this material which afforded substantial protection in mice. In 1955, Bain applied this extraction procedure to Serotype I (Roberts) P. multocida. This material was demonstrated to contain a protective protein-polysaccharide complex plus a polysaccharide which behaved as a hapten. Mukkur and Nilakantan (1969) confirmed the immunogenicity of this material in cattle. Gaunt et al. (1977) extracted Serotype 3 (P. 1059) P. multocida and found it to be immunogenic against a challenge infection of the homologous (Serotype 1 X-73) strain. Two antigenic components from each serotype were observed to show lines of identity on gel diffusion precipitin tests.

Mukkur (1977) immunized mice with a KSCN extract of Pasteurella hemolytica Serotype 1. These mice were found to resist a challenge infection with P. multocida Type A, thus demonstrating cross protection. Antisera produced in mice against P. hemolytica was bactericidal for P. multocida and vice versa. Bovine antibody against the KSCN extract of P. multocida was bactericidal for P. hemolytica. Mukkur (1979) compared the immunizing efficiency of the KSCN extract of P. multocida with formalinized P. multocida in mice. The survival rate for the mice receiving the formalinized bacterin varied from 10 to 63%. All mice receiving the KSCN extract were protected; however, this protection was not permanent. Only 40-53% of the mice survived more than one week. Apparently, better protection was obtained when the KSCN extract was combined with the formalinized cells. The KSCN extract alone produced serum with the highest bactericidal titer, compared to

the formalinized cells or a mixture of both. Immunoelectrophoresis of the KSCN extract revealed three antigenic components.

Prince and Smith (1966a) used immunoelectrophoresis to study the antigens of P. multocida. Working with a Type B strain they demonstrated the presence of 18 distinct antigenic components in various extracts. The majority of these were associated with structures other than the capsule and were designated with the letters a through n. Two antigens (α and β) were associated with the capsule and/or cell wall of the bacterium. The α component was found to be heat labile and resistant to trypsin digestion. The β antigen was heat stable and trypsin and pepsin resistant. The α antigen did not stain with Sudan black whereas the β antigen did. Both took up the protein stain thiazine red. They felt that the α antigen was analogous to the "protein" fraction of Knox and Bain (1960). The β antigen was thought to be analogous to the crude polysaccharide.

Further study (Prince and Smith, 1966b) demonstrated that 16 of the 18 soluble antigens found in avian and bovine strains were common to all the strains studied. The α and β antigens were found to be type specific; however, the α antigen would often cross-react.

Baxi et al. (1970) were able to isolate several antigens from P. multocida strains in an attempt to determine type specificity. They prepared sonicated bacteria and subsequently precipitated the sonicate with 0.5, 1.0, 1.5, and 2.0 volumes of cold 96% ethanol. They compared these fractions with the whole sonicate and with heat stable antigen prepared by autoclaving washed bacteria. Using gel diffusion

precipitation, immunoelectrophoresis, and indirect hemagglutination, they found that several distinct antigens were exposed by sonication but were unable to delineate the type specificity of these or relate them to protective effects. They did report, however, that the lipopolysaccharide of Type B was identical to that of E.

Saline and phenol-water extracts of Types B and E P. multocida were compared by Penn and Nagy (1974). The saline extract was found to contain both an endotoxin component and another component theorized to be the capsular antigen. Treatment of the saline extract with Na deoxycholate caused the depolymerization of the endotoxin. Endotoxin thus treated diffused from the antigen well more efficiently in the gel diffusion precipitin test. The phenol-water extract was found to contain both of these antigens. Based on their studies, they concluded that the α and β antigens reported by Prince and Smith (1966a) were analogous to the endotoxin and capsular polysaccharide respectively.

The ultimate complexity of the antigenic makeup of P. multocida was not exposed until the studies by Namioka and associates. Namioka and Murata (1961a, b, c) extracted what they determined to be the O or somatic antigen of P. multocida using 1N HCl. They found that the somatic antigens found in a rough strain were identical with those found in the iridescent variant of the same strain. They determined that the somatic antigens were destroyed by boiling and could be divided into two groups, common and specific. The somatic antigens were shown to be a complex of antigens produced by a combination of various factors. By means of cross absorption studies they were able

to group cultures on the basis of these somatic antigens. The chemical composition, structure, and location of the somatic antigens has not been extensively studied.

Heddleston et al. (1966) described the isolation and properties of "particulate antigens" from cold saline extracts of the cells from the gray colonies of two immunologically distinct types of P. multocida. These particulate antigens were isolated from cell-free supernatants from agar-grown cells by centrifugation at 105,000 g for two hours. The particulate antigen consisted mainly of small spherical particles with average diameters of 20-50 n. Electron microscopic observations of shadow casts showed these particles to be flattened membraneous sacs. They possessed many of the properties of endotoxins, i.e., they were toxic, high molecular weight, nitrogen-containing, phosphorylated lipopolysaccharides which were readily inactivated by mild acid treatment. These "particulate antigens" were found to be highly immunogenic in chickens, mice and rabbits. Passive protection with rabbit antisera in mice revealed good protection in one strain but not in the other.

Rebers et al. (1967) isolated a particulate lipopolysaccharide-protein complex from a virulent capsulated strain of P. multocida isolated from a case of hemorrhagic septicemia in bison (M-1404). The complex was extracted by mixing agar-grown cells in cold formalinized saline, followed by removal of the cells and centrifugation of the supernatant at 105,000 g for 2 hours. This complex was found to be both toxic and protective in mice, rabbits and calves. The gross

chemical composition and toxicity of the antigenic complex was found to be similar to that of endotoxins extracted by the Westphal or Boivin procedures. Extraction of a similar material from two avirulent noncapsulated fowl cholera strains produced a similar antigenic complex. It was capable of inducing typical signs of fowl cholera in chickens. Crutchley et al. (1967) observed that a large amount of material indistinguishable from endotoxin was liberated in broth cultures of gram negative bacteria. They found this material to be lipopolysaccharide in nature with a molecular weight of approximately 1 million. The term "free endotoxin" was proposed for this material. Working primarily with E. coli, they found this free endotoxin to have the ability to induce nonspecific resistance to infection, diarrhea, tumor necrosis, and localized and generalized Schwartzman reactions. It was also toxic in mice and pyrogenic.

They found that free endotoxin was not merely a product of cell breakdown. Rather, it was a product of vigorous cell growth in well aerated broth cultures. They hypothesized that free endotoxin was released as a result of excess production of cell wall material. Crude free endotoxin was extracted by precipitation of the cell-free supernatant with 65% W/V ammonium sulfate. This material could also be extracted by ultracentrifugation. It was found to be equal in amount to that contained in the cells themselves. Using the Ouchterlony gel diffusion technique, the free endotoxin was found to be immunologically indistinguishable from conventionally prepared endotoxins.

The crude free endotoxin was further separated by column chromatography. It was found to contain an endotoxin fraction and a nontoxic fraction. The latter material had a much smaller molecular weight. It was found to have some of the immunological but none of the toxic properties of endotoxin. Bhasin (1970) observed that a free endotoxin from P. multocida induced a high degree of active immunity in mice. The lipopolysaccharide prepared from the same strain by phenol extraction was highly toxic and relatively nonimmunogenic.

Srivastava et al. (1970) studied culture filtrates from agar-grown cultures of P-1059. They found that the immunity obtained in mice was equivalent to that produced by cell wall material. Fractionation of the culture filtrate on a Sephadex G-50 column yielded four fractions. The first fraction was found to contain the material responsible for immunogenicity in mice. It contained 63% carbohydrate, 3.0% protein and 0.1% 2-keto-3-deoxyoctulosonic acid (KDO). The heptose content ranged from 0.01 to 0.10%. The authors stated that this material is distinct from the free endotoxin studied by Heddleston et al. (1966).

In another study (Srivastava and Foster, 1977) the first fraction obtained from gel filtration was fractionated with aqueous ether to yield a "glycolipid-like" material and with phenol to yield a "lipopolysaccharide like preparation." They found that the ether-extracted material was more protective for mice than the whole first peak. When extracted with phenol, the fraction represented by the first peak lost its immunogenicity but was toxic for rabbit skin.

Rebers and Heddleston (1974) compared the serologic and immunogenic properties of free endotoxin from capsulated and noncapsulated cells of strain X-73. The gel diffusion precipitin reactions were found to be identical. The free endotoxin from the capsulated cells was able to induce immunity more rapidly than that from noncapsulated cells. Treatment of the free endotoxin with phenol resulted in a loss of immunogenicity. This indicates that a protein component is required for the induction of immunity. They hypothesized that the free endotoxin was composed, in part, of a lipopolysaccharide-protein complex. The immunologic specificity would be determined by the carbohydrate components of the lipopolysaccharide and the induction of immunity by the protein moiety. Earlier work (Rebers et al., 1967) had shown the immunogenicity of the free endotoxin to be stable to heat and formalin. Proteins complexed to lipopolysaccharides have been shown to be more stable than free proteins (Rothfield and Pearlman-Kothencz, 1969).

In comparing the free endotoxin with Westphal-extracted lipopolysaccharide, lines of identity were observed on gel diffusion precipitin tests. Both antigens reacted with homologous antiserum prepared against whole cells but the free endotoxin was cross reactive with some of 14 other serotype antisera. The Westphal lipopolysaccharide was serotype specific. The free endotoxin also gave a coalescent precipitin line with the heat stable antigen (Heddleston et al., 1972a, b) used for serotyping P. multocida of avian species (Heddleston and Rebers, 1975). The immunoelectrophoresis patterns, however,

showed a small difference in the electrophoretic mobility of the two antigens. Ganfield (1971) determined the particle diameter of the free endotoxin by electron microscopy to be 35 to 45 nm with a Stokes radius of 18 nm. A particle weight of 7.9×10^6 was determined. Ganfield et al. (1976) further purified culture filtrates by differential centrifugation and gel filtration on Sepharose 2B. Working with a gray dissociant of P-1059, three fractions were usually obtained. The peak eluted in the void volume was predominantly carbohydrate although some protein was present. This peak amounted to about 10% of the starting material and was immunogenic in mice and turkeys.

The second fraction amounted to 75 to 95% of the starting material. It contained 25 to 27% protein and 11% carbohydrate. Small amounts, 10 to 20 µg, were protective in mice and turkeys. Larger amounts were found to be lethal, however. The mean lethal dose was 195 µg for mice and 5.7 µg for 10-day-old chicken embryos. The third peak was primarily composed of proteins which gave lines of nonidentity with the second fraction on gel diffusion precipitin tests. The third peak was much less effective in inducing a protective immune response. Isolectric focusing analysis showed that all the precipitinogenic activity was found in the range of pH 3.0 to 4.0, with a peak at 3.7.

Brogden (1977) presented electron micrographs of the blebing of P. multocida. It would appear that the free endotoxin is thus an extrusion product consisting of excess cell wall material. It would be expected to be identical to the cell wall of P. multocida and exhibit similar serologic and biochemic properties. The work of

Srivastava et al. (1970) and Srivastava and Foster (1977) indicates that other materials may be present in culture filtrates which are important in the immune response.

Various subcellular antigenic materials associated with the ribosomal constituents of pathogenic organisms have been isolated and studied. Youmans and Youmans (1964a, b, c) were the first to study what they originally described as a particulate antigen from Mycobacterium tuberculosis with a structure which in many respects resembled mitochondria. On further study they determined that this highly immunogenic and protective fraction was associated with the ribosomes of M. tuberculosis (Youmans and Youmans, 1965). Treatment of this fraction with trypsin to remove protein did not affect its immunogenicity in mice, whereas treatment with RNase resulted in a 50% reduction (Youmans and Youmans, 1966b). Treatment with sodium dodecyl sulfate increased the activity of this fraction (Youmans and Youmans, 1966a). Precipitation with ethanol resulted in a preparation with immunogenic properties in mice comparable to that found in viable attenuated H37 Ra cells of M. tuberculosis (Youmans and Youmans, 1969). It was shown that the presence of an adjuvant, either natural or artificial, was required to elicit a high level of immunity (Youmans and Youmans, 1966a). They theorized that the natural adjuvant was a mycobacterial lipoprotein membrane. If these membranes were damaged, immunogenic activity was reduced; if destroyed, the immunogenic activity was lost. Under the latter circumstance, Freund's incomplete adjuvant would completely restore immunogenicity. The effect of the

adjuvant was thought to be due to the protection it afforded the ribosomes from ribonuclease present in mouse serum. It was also shown that young viable cells were a more suitable source of the ribosomal material. In 1973 Youmans and Youmans analyzed the relationship between sedimentation value and immunogenic activity of mycobacterial RNA. The immunogenic moiety of the RNA was found to sediment at 23S and have a molecular weight of approximately 450,000.

Immunogenic materials similar to those of Youmans and Youmans were prepared from Salmonella typhimurium by Venneman and Bigley (1969). The immunity obtained was found to be equivalent to that observed with live organisms and much greater than that generated by immunization with heat-killed Salmonellae, purified lipopolysaccharide, or crude and SDS treated endotoxins. Contrary to the findings of Youmans and Youmans (1966a), the immunity obtained was independent of the need for adjuvant and dependent on the dosage of the immunogen. Further study (Venneman et al., 1970) revealed the immunogenicity to be inhibited by Freund's incomplete adjuvant at 15 days but not at 30 days post-immunization. Their preparations were unaffected by treatment with trypsin, DNase, RNase or pronase plus RNase. Venneman and Berry (1971a) were able to demonstrate the passive protective effect of serum from mice immunized intraperitoneally with their preparations of S. typhimurium; however, hyperimmunization of mice by the subcutaneous route resulted in serum with no demonstrable protective effect. They were able to transfer immunity to normal mice with peritoneal cells obtained from immunized mice (Venneman and Bigley, 1971b). In

addition, such recipients were rendered capable of acting as donors of peritoneal cells that conferred demonstrable resistance on normal recipients. Johnson (1972) treated ribosomes with phenol to extract the protein associated with the immunogenic fraction of S. typhimurium. He found the resulting ribosomal RNA to be nonimmunogenic, thus suggesting that immunogenicity was a property of the protein moiety.

Smith and Bigley (1972) found that a protein-rich fraction separated from an ethanol-precipitated material by salt precipitation conferred significant protective immunity in mice when combined with polyadenylicuridylic acid.

Fogel and Sypherd (1968) described the use of 2-chloroethanol to extract ribosomal proteins from Escherichia coli. Using this method, Winston and Berry (1970) extracted ribosomal proteins from Staphylococcus aureus. Immunity conferred in mice by the ribosomal protein was significant for as long as 15 days and was responsive to a booster dose. RNase had little or no effect on the protective quality of this material but heat was detrimental.

Thompson and Snyder (1971) studied ribosomal preparations of Diplococcus pneumoniae. They found the peritoneal route of immunization to be most efficient and that mice were protected up to 12 weeks post immunization. The immunogen was sensitive to both RNase and protease.

Jensen et al. (1972), using column chromatography, were able to separate ribosomes from what they determined to be the protective antigen of Vibrio cholera. The protective antigen was a heterogenous colloid. It contained protein, lipid and carbohydrate in a ratio of

3:1:1. Further purification was unsuccessful.

Thomas and Weiss (1972) found that ribosomal material from group B Neisseria meningitidis protected mice against both a homologous and heterologous challenge. The protective immunogen was unaffected by RNase and impaired by pronase digestion.

Smith et al. (1973) described the protective effect of immunogenic ribosomal fractions of Pseudomonas aeruginosa. Protection from homologous challenge ranged from 80 to 100 percent, while no protection was afforded from heterologous challenge.

The immunogenicity of ribosomal vaccines prepared from group A Type 14 Streptococcus pyogenes was examined by Schalla and Johnson (1975). They found that optimal protection was attained by subcutaneous administration of 326 µg of protein in Freund's incomplete adjuvant. Animals immunized with higher or lower antigen concentration showed decreased survival. Mice optimally immunized against Type 14 S. pyogenes were also protected from heterologous challenge with M Types 2, 5, 8, and 12. The immunogenic moiety was determined to be protein in nature and not RNA.

Ribosomal fractions were isolated from Listeria monocytogenes by Coppel and Youmans (1969) and from Yersinia pestis by Johnson (1972). These were found to be nonimmunogenic in mice.

Baba (1977) isolated a ribosomal fraction from P. multocida strain P-1059. He purified this material by differential centrifugation followed by zonal electrophoresis on a starch block and separation on a Sephadex G-200 column. The fraction he obtained induced a high

level of immunity in mice to a homologous challenge. Treatment with sodium docecyl sulfate resulted in a 13% loss of protection and RNase caused a 60% loss of protection. Incubation for 1 hr. at 37° C caused no loss in immunogenicity. Purified protein and purified (phenol-extracted) RNA were nonimmunogenic. The supernatant fraction remaining from the original centrifugation of this material at 105,000 g for 2 hr. was not protective.

Immunization

The use of various immunizing agents against Pasteurella pneumonia in cattle has proven equivocal (Carter, 1967b). Carter (1961a) recommended that oil-adjuvant vaccine be prepared from virulent, capsulated organisms of Type A. He stated that "because Types A and D are not as antigenic as Type B, as large or larger doses" should be used to immunize cattle. Because of adverse tissue reactions, oil-adjuvanted vaccines have not been extensively employed. The protection induced by commercial bacterin products has also been called into question. Palotay et al. (1963) were able to reduce the incidence of clinical pneumonic pasteurellosis with commercial bacterins but not eliminate it. Their study was done on calves vaccinated at least one month prior to shipping.

Larson and Schell (1969) immunized recently weaned calves upon arrival at a feedlot. They employed various vaccine preparations and monitored rectal temperatures, white blood cell counts, serum titers, serum protein, and clearance of a beta-hemolytic Streptococcus from

the blood. Pasteurella bacterins were found to: 1) increase rectal temperatures to 103.5° F or higher, 2) cause a leukocytosis with neutrophilia, and 3) not change serum protein levels. This apparently did not alter the clinical appearance or affect the general state of the animal's health. Antibody titers varied greatly. All but 2 animals tested were able to efficiently clear the beta-hemolytic Streptococci. The two calves not clearing the Streptococci were unvaccinated controls. The authors believed the increased ability to clear the Streptococci was due to a nonspecific, generalized immunopotentiation caused by the endotoxin present in the bacterins.

A large amount of effort has gone into the development of vaccine preparations for hemorrhagic septicemia and avian pasteurellosis. A brief review will be presented here.

The development of an effective immunizing agent for hemorrhagic septicemia has been aided by two factors: 1) Types B and E, which are responsible for hemorrhagic septicemia, do not possess heavy mucoid capsules similar to the Type A strains (Carter, 1967b) and 2) Types B and E are more antigenic than Type A strains (Carter, 1961a) possibly because of a higher protein component in their capsules (Bain, 1955). As a result, formalin-killed bacterins, emulsified in a suitable adjuvant, have been used to establish solid immunity in cattle and buffalo (Carter, 1967b).

Many attempts have been made to immunize chickens and turkeys against P. multocida. An effective immunity against fowl cholera using aluminum hydroxide adsorbed killed vaccine was demonstrated by Heddleston and Reisinger (1960). Immunity lasted at least 52 weeks.

The demonstration of secretory antibody induced by the introduction of viral antigens in the respiratory tract of chickens led Wichmann and Stoner (1974) to introduce bacterial antigens via the same route. A 75% protection rate was observed in chickens inoculated twice with a washed whole cell formalinized bacterin. No cross protection against heterologous strains was observed however.

The use of live, attenuated P. multocida vaccines was pioneered by Pasteur in 1880. However, Pasteur's vaccine did not prove practical because uniform attenuation could not be attained. Heavy losses sometimes occurred in vaccinated flocks (Harshfield, 1965). In 1914, Hayden produced immunity in rabbits, pigeons and chickens by injecting a live attenuated P. multocida vaccine (Bierer and Scott, 1969). Heddleston and Rebers (1968) reported that active immunity could be produced in chickens with the oral administration of a killed P. multocida vaccine. Bierer and Eleazer (1968) used a live attenuated Serotype 3 P. multocida to immunize turkeys via the drinking water. This drinking water vaccine provided significant immunity when used for a 21 day period prior to challenge. Adverse vaccine effects were not observed. Bierer and Scott (1969) compared drinking water vaccine administered every 2 weeks with oil-adjuvanted bacterin and found the former highly superior. Bierer and Derieux (1972a) found the avirulent strain CS-148 produced a high degree of protection against a virulent homologous or heterologous challenge. The injection of a commercial oil-base bacterin prior to the use of the avirulent vaccine proved superior to either vaccine or bacterin used alone. The duration of

immunity was determined as sufficient for commercial application (Bierer and Derieux, 1972b); however, up to 4.2% mortality was reported from the vaccine (Bierer and Derieux, 1972a, b).

Maheswaran et al. (1973) used a high-temperature mutant (M-2283, Serotype 4) to vaccinate turkeys by drinking water, endotracheal and subcutaneous routes. Birds vaccinated by the drinking water or endotracheal routes acquired good immunity to challenge by identical routes. However, poor resistance to intramuscular challenge was observed. Turkeys vaccinated intramuscularly were less adequately protected. Protection against heterologous challenge with representative strains of Serotypes 1, 3, 5 and 9 was observed. No adverse vaccine effects were reported in this study.

Heddleston et al. (1975) were able to induce passive cross immunity in chicks and turkeys with antiserum from birds vaccinated with Serotype 12 P. multocida in the drinking water. This indicated that the cross immunity was humoral in nature. However, no agglutinins or precipitins were associated with the cross immunity. Several other serotypes were tested. Three of these were found to be virulent for 4 week old turkeys; three were not.

In 1972, Heddleston and Rebers reported on the use of a fowl cholera bacterin prepared from the liver and blood of a turkey that had died of acute fowl cholera. The tissues were homogenized in a blender with 0.5% formalinized saline and the particulate debris removed by centrifugation. Turkeys receiving two injections of this preparation exhibited 90% protection against a heterologous strain. Mice treated

similarly were not protected. Heddleston and Rebers (1974) further studied *in vivo* propagated bacterins and compared these to *in vitro* grown tissue bacterin. They also inoculated embryonating turkey eggs and chicken eggs with P-1059 and prepared formalinized bacterins from each. Other bacterins were prepared with tissues from turkeys, mice and chickens infected with P-1059. It was found that no significant cross immunity to strain X-73 could be induced in chicks with bacterins prepared from strain P-1059 grown on or in media enriched with turkey tissues. However, turkey embryo bacterin protected 59% and 93% of turkeys when challenged with strain X-73 by the intramuscular and drinking water routes, respectively. Cross immunity was slight with the chicken embryo bacterin and nonexistent with bacterins prepared from infected chicken or mouse tissues. In mice, they found in one instance, that a bacterin from infected mouse tissue induced 55% protection to heterologous challenge. Anaphylactic shock was produced in mice by a second injection of turkey and chicken embryo bacterins.

Passive protection to a heterologous strain was induced in chickens with turkey antisera prepared from infected turkey tissue (Rebers et al., 1975). Turkey antisera produced by injection of an agar-grown bacterin was not cross protective in chickens. Curiously, more antibody was present in the latter preparation. However, qualitative differences existed between the two antisera. Antigens extracted from infected tissues reacted with antisera induced in turkeys by the tissue bacterin. The IgG fraction of the cross protective turkey antisera was the major immunoglobulin fraction involved in the cross protection.

The actual mechanism of immunity to pneumonic pasteurellosis in cattle has not been extensively investigated. Many workers have attempted to equate mechanisms elucidated in poultry and mice with those in cattle. Also, attempts have been made to compare infections with Types B and E in cattle with Type A. These and other factors have tended to limit our knowledge. Most of the work to date has centered on the humoral mechanisms of immunity.

Collier et al. (1962) studied natural outbreaks of shipping fever in calves. Both P. multocida and P. hemolytica were found to be responsible for pneumonic lesions. Blood from calves in the early stages of shipping fever did not yield significant bacterial growth in tryptose broth cultures. Thus it would seem that early dissemination to tissues is not a factor in Pasteurella infection as it is in other species (Collins and Woolcock, 1976). Vardaman et al. (1962) studied the transmission of P. multocida and P. hemolytica in calves. They found that the bacteria were transmitted very effectively by contact. Thompson et al. (1975) correlated various physiological and immune parameters with clinical appearance and post mortem pneumonic lesions in beef calves suffering from pneumonic pasteurellosis. Temperatures, plasma fibrinogen levels, and mean numbers of P. hemolytica recoverable from the nasal cavity were all significantly increased in affected animals. Numbers of P. multocida in the nasal cavity were not increased over the period of the study. No significant differences in antibody titers to P. multocida, P. hemolytica or PI_3 were observed between clinically ill and normal calves. An eosinopenia

was noted in clinically ill animals.

Extensive investigations have been carried out in other species to elucidate the role of the immune system in Pasteurella infections. Carter (1964) was able to correlate passive protection to Types B and E in mice with the indirect hemagglutinating titer of rabbit antiserum. The antigen used to sensitize the human O RBC's was a phenol-extracted lipopolysaccharide. Alexander and Soltys (1973) were unable to relate serum agglutination titers to the level of protection afforded turkeys. In their study, bacterins of Types 1:A and 5:A protected turkeys; but those vaccinated turkeys that died often displayed the highest agglutinin titers. Serum with a titer of 640 passively protected turkey poults but to a lesser degree than serum with a titer of 160. They were able to passively protect mice to some extent with 5:A but not with 1:A antiserum.

Bullen et al. (1971) studied the bactericidal effects of rabbit serum on P. multocida. The authors found that specific antibody, complement, and a capacity of the serum to bind iron were all essential to stasis of bacterial growth. Addition of Fe containing compounds to the serum abolished the bacteriostatic effect. Earlier work had demonstrated that passive immunity to P. multocida could be abolished by injection of iron containing compounds.

Collins (1973) quantitated the growth of P. multocida Serotype 5:A in both normal and vaccinated mice. In normal mice challenged intraperitoneally, the bacteria were found to spread rapidly to the liver and spleen via the blood. Numbers of bacteria recovered from

the blood, liver, spleen and peritoneal cavity all rose progressively until death. Similar situations were observed in mice challenged intravenously, subcutaneously, and aerogenically although differences in the rate of dissemination from the primary site of infection were observed. In vaccinated mice challenged intravenously, bacteria were rapidly cleared from the blood. Numbers rose slightly by 6 hr. post challenge in the liver and spleen and then gradually decreased. Immunity to intraperitoneal challenge was apparently a direct result of the ability of the mouse to prevent the spread of large numbers of organisms to other tissues. Challenge of immune mice via the footpad resulted in a localization of the inoculum there also. Mice were not able to resist aerogenic challenge as easily. A 5 to 10 fold increase in viable numbers of P. multocida occurred in the lungs over a 24 hr. period in mice vaccinated with 2-3 doses of heat-killed vaccine.

In a companion study on footpad challenge (Woolcock and Collins, 1976), hyperimmune serum injected 24 hr. prior to challenge was effective in localizing the infection. The foot area of some mice developed large abscesses from which large numbers of viable P. multocida could be recovered. Removal of the draining popliteal lymph node 7 hr. prior to the injection of serum did not alter the results.

Survival data and growth curves indicated that a humorally mediated immune mechanism was involved. In the absence of specific opsonins, more than 90% of the bacteria remained in the extracellular growth phase. Immune serum was found to increase the rate of

phagocytosis and reduce the number of noncell-associated bacteria in peritoneal washouts. However, the numbers of bacteria in such washouts continued to increase. Addition of penicillin to prevent growth of bacteria outside of peritoneal macrophages did not slow the increase of viable counts. Not even peritoneal macrophages taken from demonstrably immune mice were able to inactivate a challenge inoculum in vitro over a 60 minute period. However, in a later study (Collins and Woolcock, 1976) it was seen that, in animals passively protected with immune serum, the numbers of viable bacteria increased up to 60 minutes following challenge and thereafter decreased. When suspensions of peritoneal and spleen cells were taken from immunized donors and inoculated into normal recipients, no protection was observed.

In another study on the use of adjuvants (Woolcock and Collins, 1976), resistance to aerogenic challenge was increased with bacterins incorporated into either Freund's Complete or Incomplete adjuvant. Protection in all cases was found to decrease with an increase in the challenge dose. Unlike the situation recorded in fowl cholera, normal mice housed with aerogenically infected mice did not develop an infection.

Although it has been generally recognized that immunity to P. multocida infection is primarily humoral in nature, one report of a cell mediated assay appears in the literature (Maheswaran et al., 1975). Using lymphocyte transformation adapted to microtiter plates, they demonstrated significant responses in turkeys immunized with either an

oil-adjuvanted bacterin or an avirulent live oral vaccine. They used heat-killed, washed organisms and free endotoxin (Rebers and Heddleston, 1974) as antigens. In addition they sonicated live bacteria and separated the sonicate into insoluble and soluble fractions by differential centrifugation and used these as antigens. The sonicated insoluble fraction gave the highest stimulation index followed by the sonicated soluble fraction, heat-killed organisms and free endotoxin.

Cell mediated immunity to Type 1 P. hemolytica has been assayed by measuring delayed skin reactions and macrophage migration inhibition in mice (Tizard and Ellicott, 1974). The authors were unable to demonstrate delayed skin hypersensitivity in the footpads to either lipopolysaccharide or EDTA extracted lipoprotein. An Arthus-type reaction was observed at 2-6 hours post infection, however. Migration inhibition was performed on pooled peritoneal macrophages from 5 days post infection, was 44% at ten days then returned to insignificant levels at 25 days. Inhibition of macrophage migration by the lipoprotein antigen was observed at 5 days and increased steadily throughout the observation period. At the end of the 25 day period inhibition was 80%. The authors concluded that some type of cell mediated response did occur in mice infected with P. hemolytica.

MATERIALS AND METHODS

Bacteria

Two strains of Pasteurella multocida were utilized throughout this study. The majority of the experimentation was conducted with P-2383, isolated from a case of bovine pneumonia presented to the Veterinary Diagnostic Laboratory, Iowa State University, Ames, Iowa in 1977. This isolate was culturally and biochemically typical of the species. Subsequent typing revealed it to be a Type A (Carter's typing system). The second isolate utilized was P-1062, also a Type A, kindly provided by Philips Roxane, Inc., St. Joseph, Missouri. Each strain was stored in a lyophilized form. In addition, a broth culture of each strain was inoculated into the yolk sac of 6-day-old embryonating chicken eggs. Following incubation at 37° C for 18 hr., the yolk material was aseptically removed and frozen in aliquots at -70° C. Individual aliquots were thawed and streaked on the 5% bovine blood agar plates (BAP). Following incubation at 37° C for 18 hours, individual colonies were picked for further cultivation.

Organisms were cultivated in liquid media consisting of brain-heart infusion broth[1] (BHISY) with 5% sterile normal bovine serum (NBS) and 0.5% yeast extract. Where large batch growth was undertaken, cultures were aerated in a 5% CO_2-air mixture.

Experimental cattle

The first group (Group I) consisted of four, 7-8 mo. old Holstein-

[1]Difco, Detroit, MI.

Fresian bulls weighing approximately 500 lb. These calves were maintained in stalls in a controlled-temperature environment for the duration of the experiment. The second group (Group 2) consisted of 10 crossbred Angus heifers and steers approximately 6 months of age and weighing 350-500 lbs. These animals were maintained in outdoor confinement facilities. The calves in this group were de-wormed with levamisole,[1] treated twice for coccidia with Bovo Cox,[2] given two injections of vitamins A and D_3[3] and vaccinated against blackleg.[4] None of the animals in either group exhibited any evidence of respiratory disease. Serum samples were taken upon arrival and just prior to participation in the investigation. The sera were analyzed for the presence of antibody to P. multocida by indirect hemagglutination tests.

Additional groups of cattle were studied in the early stages of this work. These animals were from herds having respiratory disease in which a Type A P. multocida was isolated. These cattle were used as a source of serum and for lymphocyte blastogenesis studies. Cattle for antiserum production were borrowed from another research project.

Animal Inoculation

Attempted sensitization of cattle was carried out by a variety of methods. Calf #5 had been injected 8 weeks previously with Freund's

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[1]Pitman-Moore, Inc., Washington Crossing, NJ.

[2]International Multifoods Corp., Minneapolis, MN.

[3]Med Tech, Inc., Elwood, KS.

[4]Fermicon 7, Bio Ceutic Laboratories, St. Joseph, MO.

complete adjuvant[1] subcutaneously. For this study, an intravenous injection containing 2 ml of a tube 10 McFarland suspension of heat-killed P-2383 mixed with 10 mg of ground Mycobacterium tuberculosis H-37 RA[1] was administered intravenously in one dose. Calf #7 received a suspension consisting of freeze-thawed viable P-2383 adjusted to tube 10 McFarland incorporated in a ratio of 1:1 in Freund's complete adjuvant. One ml of this suspension was injected subcutaneously in each of two sites in the neck. Calf #61 received a single 7 mg (protein) dose of RNA-protein incorporated in Freund's complete adjuvant; the 2 ml volume was given subcutaneously in two sites. Calves 64, 68, and 71 were given two intradermal injections of the RNA-protein ten days apart in multiple (at least four) sites. The dose varied from 4.8 to 12.0 mg on a protein basis. Calves 56, 62, and 67 were given two intradermal injections of the crude ribosome (CR) fraction at 10-day intervals in multiple sites. The dose varied from 2.9 to 6.0 mg protein. Calves 3, 55, 57, 61, and 72 received 0.5 ml of a viable whole broth culture of P-2383 intradermally in two sites at 10 day intervals. The rectal temperature of calf #3 was monitored daily, but the other calves were closely observed only. Calf #61 was included in two treatment groups, but the treatments were separated by several months. Calves 2, 5 and 64 served as controls at various times during this work.

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[1]Difco, Detroit, MI.

Production of antiserum

Antisera were produced in cattle by two methods: 1) Mature Holstein-Fresian steers were injected subcutaneously with 2 ml of heat-killed P-2383 adjusted to a density of tube #4 McFarland and incorporated in aluminum hydroxide gel.[1] Six injections were given at one week intervals and serum collected two weeks following the final injection. 2) Viable whole cultures were injected in a very low dose intradermally for two injections followed by six intravenous injections of the same material. The amount injected intravenously increased from 1 ml to 5 ml of a heavy growth given at 3 day intervals. This procedure resulted in an increase in rectal temperature and rapid respiration subsequent to each injection. The calf was bled two weeks following the last injection.

A third antiserum used in this work was provided by Philips Roxane. The serum was obtained from cattle hyperimmunized with live Pasteurella multocida P-1062.

Bacterial fractions

Potentially antigenic fractions were isolated from P. multocida by a variety of procedures.

Crude Ribosomes (CR) and Ribosomal Supernatant (SR). Crude ribosomes were isolated from P-2383 grown for 5-6 hours in BHISY and harvested

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[1]Mann Research Laboratories, Inc., New York, NY.

by centrifugation at 10,000 g for 30 minutes at 5° C. Cells were washed three times in sterile 0.15 M phosphate buffered saline (PBS) pH 7.4 and resuspended in cold sterile 0.01 M Tris-HCl buffer (Tris[hydroxymethyl] aminomethane)[1] containing 5 X 10^{-3} M $MgCl_2$. The cells were disrupted in a Ribi Cell Fractionator[2] with a nitrogen cooled valve at 40,000 to 60,000 Psi and collected in a cold slush of Tris buffer. Cellular debris and whole cells were removed by centrifugation at 27,000 g for 1 hr. at 5° C. The supernatant fluid was centrifuged at 65,000 g for 1 hr. at 5° C. The pellet was discarded and the supernatant fluid passed through a 0.45 µm sterile Falcon filter.[3] The filtrate was centrifuged at 108,000 g for 3 hr. at 5° C. The supernatant fluid was aspirated and saved. The pellet was gently resuspended in Tris-HCl-$MgCl_2$ buffer. The pellet constituted the crude ribosomes (CR) and the supernatant fluid was designated (SR) The CR and SR fractions were stored in aliquots at -70° C. In addition, the SR fraction was concentrated 16-fold in a dialysis casing by application of Acquacide II[4] to the exterior of the casing. The concentrated SR was then applied to a column containing Sephadex G-200[5] in a .02 M Tris buffer with 1% sodium azide to retard bacterial

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[1]Sigma Chemical Co., St. Louis, MO.

[2]Ivan Sorvall, Inc., Newtown, CT.

[3]Falcon Plastics, Los Angeles, CA.

[4]Calbiochem, LaJolla, CA.

[5]Pharmacia Fine Chemicals, Uppsala, Sweden.

growth. Fractions were collected and analyzed for protein content by the Bio-Rad assay[1] and absorbance at 260-280 MM. The fractions were analyzed in the gel diffusion precipitin test against bovine anti-P-2383 antiserum.

Crude ethanol precipitated RNA-protein

Crude ethanol precipitated RNA-protein was prepared by a modification of the method of Smith and Bigley (1972). The bacteria were processed by the same procedure used for isolation of the CR and SR fraction up to and including the filtration following the 65,000 g centrifugation. The filtrate was brought to 0.1 M sodium concentration by the addition of NaCl. Two volumes of cold (-20° C) 95% ethanol were added dropwise in the cold. The mixture was left at -20° C for 24 hr. to allow precipitation of the nucleic acids and protein. The mixture was centrifuged at 10,000 g for 10 min at 0° C. The supernatant fluid was discarded and the precipitate allowed to dry by evaporation at -20° C. The precipitate was resuspended in a minimal amount of Tris-HCl-$MgCl_2$ buffer and stored at -70° C. Aliquots of this material were dried *in vacuo* and stored at -20° C.

For use in lymphocyte transformation, the CR, SR and RNA-protein fractions were diluted in Medium-199 (M-199)[2] containing 5 X 10^{-3}

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[1]Bio-Rad Laboratories, Richmond, CA.

[2]GIBCO, Grand Island, NY.

M $MgCl_2$. Aliquots of diluted material were frozen at -70° C and fresh aliquots were used daily.

Ribosomal protein

Ribosomal protein was extracted by the method of Fogel and Sypherd (1968). Briefly, P-2383 was grown, washed and disrupted as for production of the CR fraction. The cellular disintegrate (200 ml) was precipitated with 42 grams of solid ammonium sulfate $(NH_4)_2SO_4$ and centrifuged at 25,000 g for 10 min. at 0° C. Another 42 grams of $(NH_4)_2SO_4$ was stirred into the supernatant fluid and mixed for 3 min. at 0° C and the pellet resuspended in 200 ml of Tris-HCl-$MgCl_2$ buffer. To the resuspended pellet, 42 g of $(NH_4)_2SO_4$ were added followed by centrifugation. An additional 77 g of $(NH_4)_2SO_4$ were added to the supernatant fluid. Following centrifugation the last two pellets containing crude ribosomes were resuspended in Tris-HCl-$MgCl_2$ buffer and combined. These were analyzed for protein and RNA content. To extract ribosomal protein, 2.3 ml of the crude $(NH_4)_2SO_4$ precipitated ribosomes were mixed with 5 volumes of 2-chloroethanol[1] in an ice bath at 0° C. HCl was added to a final concentration of 0.06 N. The mixture was held at 0° C for 2 hr. with periodic mixing in a hand homogenizer. It was centrifuged at 5000 g for 10 min. at 0° C and the supernatant fluid dialyzed against several changes of PBS (pH 7.4) for 72 hr. at 5° C. The dialysate was centrifuged at 10,000 g for

[1]Fisher Scientific, Fairlawn, NJ.

20 min. The pellet was suspended in 5 ml Hanks balanced salt (HBSS)[1] and protein and RNA content analyzed by the Bio-Rad and Orcinol procedures respectively.

Saline extracted capsule

Saline extracted capsular antigen was prepared by a modification of the method of Penn and Nagy (1974). Bacteria were grown on dextrose starch agar[2] and incubated for 18 hr. at 37° C. They were washed off the agar with 2.5% (W/V) sodium chloride. The resulting suspension was centrifuged at 27,000 g for 1 hr. The supernatant fluid was passed through a 0.45 μm membrane filter, dialyzed for 3 days against several changes of distilled water at 5° C and freeze-dried to yield the saline extract.

Phenol extracted endotoxin

Phenol extracted endotoxin was prepared by a modification of the method of Westphal and Jann (1965). Cells were grown in BHISY for 18 hr. and harvested by centrifugation at 8,000 g for 30 min. The cells were washed three times in sterile PBS (pH 7.4) and resuspended in sterile distilled water. The cells were mixed for 8 min. at medium setting in a Waring Blender with 2 volumes of

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[1] GIBCO, Grand Island, NY.

[2] Difco, Detroit, MI.

redistilled phenol at 25° C. The material was cooled to 20° C and centrifuged at 4000 g for 20 min. The aqueous phase was removed and the remainder re-extracted with a volume of distilled water equal to the volume of the original cell suspension. The aqueous phases were combined and dialyzed exhaustively against distilled water at 5° C. The material was concentrated in a dialysis casing using Acquacide II followed by centrifugation at 108,000 g for 2 hours. The pellet was resuspended in PBS and stored frozen at -70° C.

Trichloroacetic acid-extracted antigen

The trichloroacetic acid (TCA) extraction was performed by a modification of the method of Boivin and Mesrobeanu (1935). P-2383 was grown in BHISY at 37° C for 18 hours. The bacteria were concentrated by centrifugation at 8000 g for 30 min. at 5° C and washed three times in PBS. The cells were resuspended in PBS and an equal volume of cold 0.5 N trichloroacetic acid was added with mixing. The temperature of the mixture was maintained at 5° C for 3 hours. It was warmed to 25° C and centrifuged at 8000 g for 30 min. The supernatant fluid was brought to pH 6.5 by the addition of NaOH and cooled to 0° C. Two volumes of 95% ethanol at -20° C were added and the mixture was allowed to precipitate overnight at -20° C followed by centrifugation at 4000 g for 30 min. at -4° C. The precipitate was dissolved in a minimal amount of distilled water and dialyzed against 8 changes of distilled water for 4 days. The dialysate was centrifuged at 30,000 g to remove any debris. The antigen complex was pelleted by

centrifugation at 105,000 g for 2 hr. at 5° C. The precipitate was suspended in a minimal amount of distilled water. This material was stored frozen at -20° C and diluted in M-199 for use in lymphocyte transformation.

Heat-extracted capsule

Heat-extracted capsular material was prepared from broth-grown cultures of P-2383 and P-1062. The bacteria were grown in 250 ml BHISY, washed three times in PBS, pH 7.3 and resuspended in 10 ml of PBS. They were heat-killed in a water bath at 60° C for one hour and centrifuged for 30 min. at 4000 g to remove intact cells. The supernatant fluid was dialyzed for 3 days against 6 changes of distilled water at 5° C and frozen in aliquots at -70° C. For use in lymphocyte transformation, the heat-extracted capsule of P-2383 was suspended in M-199. The heat extracted capsules of both P-2383 and P-1062 were utilized in the indirect hemagglutination tests.

Crude polysaccharide

Crude polysaccharide was prepared from spent media remaining after growth of P-2383 in BHISY and following centrifugation to remove the bacteria. A 10% solution of hexadecyl trimethylammonium bromide (Cetavlon)[1] in distilled water was added to the spent media at a rate of 10 ml per liter of media. The mixture was stirred at 5° C

[1]Eastman Chemical Corp., Rochester, NY.

for 6 hr. and then allowed to stand without stirring for 24 hours. The precipitate was collected by centrifugation at 8000 g for 20 min. The tan, gelatinous pellet which resulted was resuspended in 0.9 M $CaCl_2$ and mixed at medium speed on a Virtis homogenizer until all material was in suspension. It was warmed to 56° C and held there for 1 hr. and then cooled to 5° C. The nucleic acids were precipitated by the addition of cold absolute ethanol (-20° C) to a final concentration of 25%. This was followed by centrifugation for 30 min. at 20,000 g and 0° C. The supernatant fluid was treated with 1.5 volumes of absolute ethanol at -20° C followed by centrifugation at 10,000 g for 30 min. at 0° C. The supernatant fluid was poured off and the pellet allowed to dry at -20° C. The dried pellet was resuspended in distilled water and homogenized. It was warmed to 56° C for 3 hr. to enhance its solubility in water and then dialyzed against four changes of distilled water. The crude polysaccharide was concentrated to 1/3 its original volume with Acquacide II applied to a dialysis casing. Any precipitated material was removed by centrifugation at 20,000 g for 30 min. followed by filtration through a Whatman #31 filter. The resulting material was designated crude polysaccharide and stored in aliquots at -20° C. Samples were diluted in M-199 for use in lymphocyte transformation.

Fractions of bacteria grown in M-199

P-2383 was also grown in M-199 at 37° C for 24 hours. The light growth obtained was centrifuged at 27,000 g for 1 hr. to pellet the

bacteria. The supernatant fluid was stored in aliquots at -20° C. Dilutions of this material in M-199 were used directly in lymphocyte blastogenesis. The bacterial pellet was heated for 1 hr. at 60° C. Five volumes of M-199 were added and the mixture centrifuged at 10,000 g for 20 min. Dilutions of the supernatant fluid were stored at -30° C and used in lymphocyte blastogenesis. The whole cells remaining in the pellet were re-suspended in M-199, stored and used identically to the two previous fractions.

Free endotoxin

Free endotoxin was isolated from the spent media of a 10-12 hr. growth of P-2383 in BHISY. Bacteria were separated from the medium by filtration in a cassette filtration system[1] and the supernatant fluid concentrated in the same system using a filter with a lower molecular weight cutoff of 100,000 daltons. The concentrated material was centrifuged at 108,000 g for 2 hr. at 5° C. The pellet was resuspended in .01 M Tris-HCl buffer and washed twice. The resulting pellet was frozen at -70° C and lyophilized. For use in lymphocyte blastogenesis, the material was resuspended in M-199.

Analysis of bacterial fractions

Antigens utilized in this work were analyzed for dry weight, protein, carbohydrate and RNA. Analysis for dry weight was performed

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[1]Millipore Corporation, Bedford, MA.

on 1) lyophilized samples or 2) aliquots of material dried in an oven at 75° C for 48 hours. Protein was estimated using 1) the Bio-Rad Protein Assay[1] with bovine serum albumin (Cohn fraction V)[2] as a standard, and/or 2) absorbance at 260-280 nm.

Carbohydrate was analyzed by the phenol-sulfuric acid method (Dubois et al., 1956) using glucose as a standard. RNA analyses were performed by: 1) the orcinol method using yeast ribonucleic acid,[2] and/or 2) absorbance at 260-280 nm. A nomograph[3] was utilized to estimate protein and RNA content from the absorbance readings at 260-280 nm.

Serological tests

Gel diffusion precipitation Gel diffusion precipitin tests were carried out in 1% agarose[4] gel in PBS containing 3 mg sodium azide per ml to retard bacterial growth. Antigens and antisera were placed in appropriate wells and incubated in a moist environment at room temperature for 3 days. Immunoelectrophoresis was carried out in commercial immunoelectrophoresis plates (IEP system).[5] Antigens were added to wells and electrophoresed at 8 ma/plate for 20 min. Sera were added to the troughs and the plates incubated in a moist

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[1]Bio-Rad Laboratories, Richmond, CA.

[2]Sigma Chemical Co., St. Louis, MO.

[3]California Corporation for Biochemical Research, Los Angeles, CA.

[4]Schlesinger Chemical Mfg. Corp., Carle Place, NY.

[5]ILC Scientific, Fountain Valley, CO.

environment at room temperature for 48 hours. Following this, they were soaked in 0.85% saline for 18 hr. and in distilled water for 2 hours. Films were stained 5 min. with Amido Black 10B, rinsed 3 min. in 5% acetic acid, and dried at 75° C in a drying oven. When dry, the films were rinsed in 5% acetic acid and cleared in fresh 5% acetic acid for 20 min. The films were dried and examined.

Indirect hemagglutination tests Indirect hemagglutination was used to quantitate antibody to various fractions of P-2383 and P-1062. Briefly, sheep red blood cells (SRBC) were washed three times in .01 M PBS pH 7.2. To 0.1 ml of SRBC, the proper amount of antigen was added and the total volume brought to 2.0 ml with PBS. Two tenths ml of a 1% glutaraldehyde[1] solution in PBS were added with mixing. The suspension was incubated at room temperature for 30 min. with periodic mixing. The SRBC were washed three times in PBS and resuspended in PBS containing 1% normal rabbit serum (PBSR) which had been inactivated by heating at 56° C for 30 min. and absorbed with an equal volume of washed SRBC. Sera to be tested were heat-inactivated and absorbed with washed SRBC. Dilutions of sera were made in PBSR in microtiter plates.[2] One drop of antigen-sensitized SRBC was added to each well. Titers are recorded as the reciprocal of the highest dilution showing agglutination.

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[1]Sigma Chemical Co., St. Louis, MO.

[2]Linbro, Flow Laboratories, Inc., Hamden, CT.

Lymphocyte blastogenesis

For the performance of lymphocyte transformation, venous blood was collected aseptically in acid-citrate-dextrose solution (ACD). ACD solution was prepared by mixing 22.0 g trisodium citrate, 8.0 g citric acid and 25.0 g dextrose in 500 ml tissue culture grade water. The solution was sterilized by autoclaving at 121° C for 17 min. and stored at 5° C. The blood was diluted 1:2 with 0.01 M PBS (pH 7.3) and layered over Histopaque-1077[1] in 50 ml siliconized glass tubes. The tubes were centrifuged at 500 g for 45 min. The buffy coat cells were removed and washed twice in HBSS without Ca^{++} and Mg^{++}. The cells were counted and a Wright's-stained smear examined for a differential count. The lymphocytes were standardized to a concentration of 2 million per ml with addition of M-199 containing 25 mM Hepes, Earles salts, L-glutamine, 5% fetal bovine serum[2] (FCS), $NaHCO_3$, 100 U/ml procaine penicillin G and 100 µg/ml dihydrostreptomycin sulfate.[2] One-tenth ml (200,000 lymphocytes) was added to each well of a plastic tissue culture multi-well plate.[3] Mitogens or antigens diluted to the proper concentration in M-199 were added dropwise in 0.025 ml quantities to triplicate wells.

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[1]Sigma Chemical Co., St. Louis, MO.

[2]Grand Island Biological Co., Grand Island, NY.

[3]Flow Laboratories, Inc., Hamden, Conneticut.

Phytohemagglutinin-P[1] and concanavalin A[2] served as mitogens to insure the immunological competence of the lymphocyte cultures. The plates were incubated at 37° C in a moist environment containing approximately a 5% CO_2: Air mixture. After 96 hr., each well was pulsed for 18 hr. with 0.25 µCi tritiated thymidine.[3] Cells were harvested with a multiple well harvester[4] and collected on filter pads. The pads were dried, placed in vials containing scintillation fluid and counted in a liquid scintillation counter.[5]

Stimulation indices were calculated by dividing the mean number of counts for each mitogen or antigen treatment by the mean of the control well counts. The $\log_{10}$ of the stimulation indices was also calculated. Statistical analysis was performed on the $\log_{10}$ values by the analysis of variance procedure (Snedecor and Cochran, 1967).

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[1]Difco, Detroit, MI.

[2]Miles Laboratories, Inc., Kankakee, IL.

[3]New England Nuclear, N. Billerica, MA.

[4]Flow Laboratories, Inc., Rockville, MD.

[5]Packard Instrument Co., Des Plaines, IL.

RESULTS

Table 1 summarizes the chemical characterization of the antigen preparations of P. multocida utilized in this investigation. The antigen preparations were analyzed for protein, carbohydrate and ribonucleic acid (RNA). The quantity of material obtained is listed on a dry weight basis and presented either as milligrams per milliliter or total yield per liter of whole broth culture. The percent protein in each fraction was measured by two separate methods: 1) Bio-Rad Protein Assay (BR) with bovine serum albumin (Cohn fraction V) as a standard and 2) by absorbance at 260-280 nm. Neither procedure was completely satisfactory and large discrepancies were noted. Given the diverse nature and complexity of many of the preparations, no one assay may be suitable for all preparations. The RNA content of the fraction was measured by two methods: 1) orcinol assay and 2) absorbance at 260-280 nm. Large discrepancies also existed between these two assays and neither one was valid for all the fractions. Total carbohydrate was measured for each fraction by the phenol-sulfuric acid method using glucose as a standard. Where replicate preparations were made, values given are mean values for the replicates.

TCA extraction of P-2383 resulted in a very small yield of material. Because of the extremely small yield, this antigen was not fully characterized and was only used in lymphocyte transformation experiments. Ten-fold dilutions of the concentrated material obtained by resuspension of the ultracentrifuged extract were made. The material

Table 1. Concentration, chemical composition and yield of isolated fractions of Pasteurella multocida. Where applicable, dry weights were obtained by drying a tared sample in an oven at 75° C for 48 hours. Protein was analyzed by 1) Bio-Rad Protein Assay using bovine serum albumin (Cohn fraction V) as a standard and 2) Absorbance at 260-280 nm. RNA measurement was performed by 1) orcinol assay using yeast ribonucleic acid as a standard and 2) absorbance at 260-280 nm. Carbohydrate was analyzed by the phenol-sulfuric acid method using glucose as a standard. The average yield per liter of broth culture was calculated for each antigen except the P-2383 saline-extracted capsular antigen. This was prepared from cells grown on solid media. The figure given for this antigen represents the average yield per Roux flask.

Fraction	Concentration mg/ml	Percent Protein		Percent RNA		Percent Carbohydrate[a]	Yield[b]
		Bio-Rad	260-280	Orcinol	260-280		
CR #3	26.6	33.8	150.0	13.0	48.8	8.5	80
RP #4	80.0	32.0	140.0	15.5	35.0	9.7	500
SR	8.9	31.0	31.0	36.0	10.0	9.5	300
R-Protein		90.0		0.1			27
Crude Polysaccharide	5.9	13.6	34.0	59.0	3.0	22.0	30
Endotoxin (Phenol)	6.5	3.1	51.0	50.8	4.0	27.3	10
Endotoxin (Free)		32.3	106.0	2.0	6.2		
Capsule (Saline X)		6.0	5.7	20.0	9.8		15[c]
Capsule (Heat X)	10.1	5.9	32.0	13.9	2.9	7.9	280
Capsule (Heat X)[d]	6.1	6.5	34.0	88.0	8.4	20.0	100

[a]Phenol-sulfuric acid method

[b]mg dry weight per liter medium

[c]mg dry weight per Roux flask

[d]Pasteurella multocida 1062

was found to suppress the incorporation of tritiated thymidine into cultures of bovine peripheral blood lymphocytes at dilutions of 1:10 to 1:100. In no instance was the material observed to stimulate lymphocytes in in vitro culture.

Preparation from M-199-grown bacteria

P-2383 was found to grow in M-199 and produce a slight turbidity. This amounted to approximately 10^8 viable bacteria per ml. Centrifugation of this bacterial suspension resulted in a substantial pellet and a clear supernatant. Heating the pellet at 60° C for 1 hr. followed by addition of M-199 and centrifugation yielded a smaller pellet and a turbid, viscid supernatant. Dilutions of the two supernatants and the washed pellet from the heat killed bacteria were made in M-199, The use of these materials in lymphocyte blastogenesis in early stages of the investigation resulted in no stimulation. High concentrations were observed to be toxic. These preparations were not studied further as they offered no advantage over other antigenic preparations.

In the preparation of subcellular fractions (CR, SR and RNA-protein) of P-2383, concentration of the live bacteria by centrifugation resulted in a loose easily disrupted pellet. High speed centrifugation up to 30,000 g was found to be necessary to concentrate the bacteria to a relatively small volume. A true pellet was not obtained even at this speed. Several batches of material were prepared. Fractionation of the bacteria in the Ribi Cell Fractionator resulted

in a light gray to amber material. Considerable foaming of the fractionated material was always observed. Examination of gram-stained smears of this material revealed cellular debris and a quantity of intact bacteria. Centrifugation of the fractionated material at 27,000 g resulted in a gray-amber supernatant and a white gelatinous pellet. The pellet was extracted with phenol by the Westphal procedure to yield a lipopolysaccharide preparation.

The difficulty experienced in pelleting viable P. multocida necessitated some means to ensure the absolute absence of viable bacteria in any of the subcellular fractions. Filtration through a 0.45 μm filter was performed with difficulty. Centrifugation of the supernatant at 65,000 g for 1 hr. removed a thick gummy material which was presumed to be DNA. The supernatant from this centrifugation readily passed through a sterilizing filter. Centrifugation of the filtrate at 108,000 g for 3 hours resulted in a relatively clear to yellow supernatant and a tan, gummy pellet. The supernatant material was designated SR and the crude ribosome pellet was designated CR. Following an overnight soaking in Tris HCl-$MgCl_2$ buffer at 5° C, the pellet (CR) was resuspended by gentle pipetting.

An average total yield of 80 mg/liter of broth culture on a dry weight basis was obtained for the CR fraction. This preparation contained approximately 34% protein (BR), 13% RNA (orcinol) and 8.5% carbohydrate. This preparation is assumed to have large quantities of material which were not measurable by the given assays. Measurement of protein and RNA at 260 and 280 nm was obviously not valid as a

reading of 150% of the dry weight was obtained.

The supernatant from the ribosomes (SR) which remained following centrifugation at 108,000 g yielded approximately 300 mg (dry weight) per liter of whole broth culture. It contained 31% protein by both the Bio-Rad and absorbance assays. The RNA content was found to be 10% (absorbance) or 36% (orcinol). The orcinol assay is known to be invalid in the presence of bacterial polysaccharide. The SR fraction was composed of 9.5% carbohydrate.

A 40 ml (94 mg protein) quantity of the SR fraction was concentrated in a dialysis casing to 2.5 ml using Aquacide II. The resulting concentrate was applied to a Sephadex G-200 column and fractions collected in 40 drop quantities. Aliquots were examined for absorbance at 260 and 280 nm. It was observed that a somewhat uniform distribution of the components of the SR fraction occurred. When applied to the gel diffusion precipitin test however, two distinct antigens were observed to be present. These antigens came off the column close together but were readily separable. The first antigen to come off was composed of approximately 9 mg protein. It gave a single sharp band in gel diffusion precipitation. The second antigenic fraction contained approximately 12 mg protein and gave a more diffuse band in gel diffusion precipitation. Immunoelectrophoresis of the concentrated SR material revealed, however, that the SR fraction actually contained three distinct antigenic moieties and not the two revealed on gel diffusion precipitation. Further attempts to separate these components were not made.

The RNA-protein material was prepared from the disrupted bacterial mass following centrifugation at 65,000 g for 1 hr. The RNA-protein fraction was obtained in a yield of 500 mg (dry weight) per liter of broth culture. It contained between 25% and 40% protein (BR), 15% (Orcinol), and 10% carbohydrate. Absorbance at 260-280 nm was observed to be inaccurate for a protein measurement. Lyophilization of the RNA-protein material yielded a pellet which was readily soluble in warm distilled water.

Ribosomal protein

Extraction of crude ammonium sulfate precipitated ribosomes with 2-chloroethanol and subsequent dialysis of the supernatant resulted in a flocculent precipitate. Upon centrifugation to pellet the flocculent material, it was found that the pellet contained essentially all of the protein associated with the original ribosomes and a trace amount of RNA. The supernatant was devoid of both protein and RNA.

A total yield of 27 mg protein (BR) per liter of broth culture was obtained with the ribosomal protein. All attempts to resolubilize the protein material were unsuccessful. It was therefore deemed to be of little value in lymphocyte transformation, immunoelectrophoresis, and gel diffusion precipitation.

P-2383 crude polysaccharide

P-2383 crude polysaccharide was recovered from the spent media of broth cultures of P-2383 in a yield of 30 mg (dry weight) per liter.

This material was composed of 13.6% protein (BR) or 34% protein absorbance. RNA content was 34% (absorbance) or 50% (orcinol). Total carbohydrate was 22%.

P-2383 heat-extracted capsule

The heat-extracted capsule of P-2383 was recovered in a yield of 280 mg (dry weight) per liter of broth culture. Both this and the heat-extracted capsule of P-1062 were a creamy white in color. It was composed of 5.9% protein (BR) or 32% protein (absorbance). RNA content was 14% (orcinol) or 2.6% (absorbance). Total carbohydrate was 7.9%.

P-1062 heat-extracted capsule

P-1062 heat-extracted capsule of P-1062 was recovered at a rate of 100 mg (dry weight) per liter of broth culture. It contained 6.5% protein BR) or 34% protein (absorbance). The RNA content was 88% (orcinol) or 8.4% (absorbance). Total carbohydrate was 20%.

P-2383 phenol-extracted endotoxin

P-2383 phenol extracted endotoxin was recovered at a rate of 10 mg (dry weight) per liter of broth culture. Total protein was 3.1% and 51% as measured by the Bio-Rad Assay and absorbance at 260-280 nm respectively. RNA content was 51% (orcinol) or 3% (absorbance). It must be presumed that the absorbance readings for protein content are invalid for this preparation. Attempts to use lyophilization instead of ultracentrifugation to concentrate this fraction led to

an inferior preparation. The lyophilized material was poorly soluble, essentially nonreactive in IHA tests, and completely non-reactive in gel diffusion precipitation.

P-2383 saline-extracted capsule

The saline-extracted capsule of P-2383 was recovered at a rate of approximately 15 mg (dry weight) per Roux flask. It was somewhat insoluble following lyophilization. Resolubilization was accomplished by suspension in saline, HBSS, or M-199 followed by incubation at 37° C for 5-18 hr. This preparation was approximately 6% protein (BR and absorbance). The RNA content was 20% (orcinol) or 9.8% (absorbance).

P-2383 free-endotoxin

P-2383 free endotoxin was obtained in a yield of 50 mg (dry weight) per liter of broth culture. This preparation was a light brown in color reminiscent of the BHISY in which it was grown. It appears that significant amounts of media constituents may have contaminated it. It contained 32% protein (BR). Protein content was 106% by absorbance which is obviously invalid. RNA content was 2.0% (orcinol) or 6.2% (absorbance)

Indirect hemagglutination

The antigenic preparations found to be of use in the indirect hemagglutination test were: 1) P-2383 phenol-extracted endotoxin, 2) heat-extracted capsular material from P-2383 and P-1062, 3)

P-2383 SR and 4) P-2383 crude polysaccharide.

1) Phenol-extracted endotoxin from P-2383 was readily coupled to SRBC by treatment with 1% glutaraldehyde. A 260 µg (dry weight) quantity suspended in 2.0 ml optimally sensitized SRBC. The results of this test were read at 2 hr. and again at 18 hours. The agglutination patterns were very stable with this antigen preparation and no significant differences were observed between the two readings.

2) The heat-extracted capsular antigen from P-2383 optimally sensitized SRBC at a concentration of 4.0 mg (dry weight) in a reaction volume of 2 ml. The heat-extracted capsule of P-1062 was optimally sensitizing at a concentration of 0.6 mg (dry weight). Both antigens coupled readily to SRBC when 1% glutaraldehyde was used as a coupling agent. Agglutination patterns were read at 2 hours. This gave the most distinct pattern for endpoint determinations. By 18 hr. the titers had decreased significantly and patterns were difficult to interpret.

3) The P-2383 SR antigen optimally sensitized SRBC in quantities of 2.0 to 2.3 mg (Protein basis, BR assay) in a reaction volume of 2.0 ml. Increased concentrations of this material resulted in a significantly decreased titer caused by hemolysis of the SRBC. The results of the IHA test with the SR antigen were read at 2 hours. Significant decreases in titer were observed at 18 hours.

4) Crude polysaccharide optimally sensitized SRBC at a concentration of 3.5 mg in a 2.0 ml reaction volume. Agglutination was

read at 2 and 18 hours. Either the sensitivity of SRBC sensitized with this antigen was poor or little antibody is formed in cattle against it.

Attempts to sensitize SRBC with other antigenic preparations

Lyophilized preparations of P-2383 saline-extracted capsule, P-2383 free endotoxin and P-2383 phenol-extracted endotoxin were found to be unsatisfactory for the IHA test. An occasional low titer was observed with hyperimmune sera.

Attempts to use the P-2383 CR and RNA-protein preparations in the IHA test were unsuccessful. A low titer was observed with hyperimmune sera. Significant hemolysis of SRBC occurred when these preparations were added to the reaction mixture. For this reason, experimentation with these fractions for use in indirect hemagglutination was limited.

Indirect hemagglutination titers of hyperimmune cattle sera

The indirect hemagglutination titers of the hyperimmune sera used in this investigation are presented in Table 2. The three sera utilized throughout this investigation were produced in cattle by: 1) multiple intravenous injections of viable P-2383, 2) multiple subcutaneous injection of heat-killed P-2383 incorporated in aluminum hydroxide gel, and 3) multiple intravenous injections of viable P-1062 The titers are expressed as the inverse of the highest dilution of serum giving significant agglutination.

Repeated intravenous inoculation of calf #3 with viable cultures of P-2383 resulted in relatively high titer serum. A titer of 320 was

Table 2. Indirect hemagglutination titers of hyperimmune sera against antigens isolated from Pasteurella multocida. Sera were produced by 1) multiple intravenous injection of viable P-2383, 2) multiple subcutaneous injection of heat-killed P-2383, and 3) multiple intravenous injection of viable P-1062. Sheep red blood cells were sensitized with: 1) P. multocida P-2383 heat-extracted capsule, 2) P-1062 heat-extracted capsule, 3) P-2383 SR antigen, 4) P-2383 phenol-extracted endotoxin, and 5) P-2383 crude polysaccharide. Titers are presented as the inverse of the highest dilution showing positive agglutination. Values represent the highest titers attained by the respective immunization procedures.

Immunization	P-2383 heat-extracted capsule	P-1062 heat-extracted capsule	P-2383 SR antigen	P-2383 phenol-extracted endotoxin	P-2383 crude poly-saccharide
Viable P-2383 intravenously	320	1280	5120	320	40
Heat-killed P-2383 in aluminum hydroxide gel subcutaneously	80	640	160	80	0
Viable P-1062	80	160	160	80	10

obtained against SRBC sensitized with P-2383 heat-extracted capsule, 1280 against P-1062 capsule, 5120 against P-2383 SR antigen, and 320 against P-2383 phenol-extracted endotoxin. Sheep red blood cells sensitized with P-2383 crude polysaccharide gave a titer of 40 against hyperimmune serum generated with viable P-2383.

Hyperimmune serum produced by subcutaneous injection of heat-killed P-2383 in aluminum hydroxide gel was of lower titer than that generated by the intravenous inoculation of viable P-2383. Titers of 80, 640, 160, and 80 were observed when P-2383 heat-extracted capsule, P-1062 heat-extracted capsule, P-2383 SR antigen, and P-2383 phenol-extracted endotoxin were used to sensitize SRBC, respectively. No indirect hemagglutination was observed with SRBC sensitized with P-2383 crude polysaccharide.

Hyperimmune serum produced commercially by intravenous inoculation of viable whole broth cultures of P-1062 presented the lowest titers observed of the three hyperimmune sera. Titers of 80, 160, 160 and 80 were observed when P-2383 heat-extracted capsule, P-1062 heat-extracted capsule, P-2383 SR antigen, and P-2383 phenol-extracted endotoxin respectively were used to sensitize SRBC. A titer of 10 was observed with P-2383 crude polysaccharide sensitized SRBC.

Indirect hemagglutination titers of sera from Group 1 calves before and after immunization are presented in Table 3. Preimmunization titers for each calf are compared to the highest postimmunization titers obtained from bleedings within 30 days following antigen administration. Titers are presented as the inverse of the highest dilution of serum showing positive agglutination. Following an overnight soaking in Tris HCl-$MgCl_2$ buffer at 5° C, the pellet (CR) was resuspended by gentle

Table 3. Indirect hemagglutination titers of sera from Group I cattle before and after immunization. Titers are given as the inverse of the highest dilution of serum demonstrating positive agglutination. Values represent the highest titer of two serum samples obtained prior to immunization compared to the highest titer of 8 serum samples obtained during a 30 day period following immunization. Calf #2 served as an unvaccinated control. Calf #3 was injected twice intradermally with viable Pasteurella multocida P-2383. Calf #5 was injected with Freund's complete adjuvant subcutaneously followed 2 months later by an intravenous inoculation of heat-killed P-2383 mixed with ground Mycobacterium tuberculosis H-37RA. Calf #7 was injected once subcutaneously with freeze-thawed P-2383 in Freund's complete adjuvant. Calf #2 served as a control throughout the investigation. At no time was this calf observed to have a titer against any of the antigens.

		TEST ANTIGENS				
Animal	Serum	P-2383 heat-extracted capsule	P-2383 SR antigen	P-1062 heat-extracted capsule	P-2383 phenol-extracted endotoxin	P-2383 crude polysaccharide
Calf #2	Control	0	0	0	0	0
Calf #3	Pre-immunization	0	0	10	0	0
	Post-immunization	0	40	10	0	0
Calf #5	Pre-immunization	0	0	0	0	0
	Post-immunization	0	0	0	0	0
Calf #7	Pre-immunization	0	0	0	0	0
	Post-immunization	0	0	0	0	0

pipetting.

Calf #2 served as an unvaccinated control. Calf #3 was injected twice intradermally with viable Pasteurella multocida P-2383. Calf #5 was injected with Freund's complete adjuvant subcutaneously followed 2 months later by an intravenous inoculation of heat-killed P-2383 mixed with ground Mycobacterium tuberculosis H-37 RA. Calf #7 was injected once subcutaneously with freeze-thawed P-2383 in Freund's complete adjuvant.

Viable Pasteurella multocida P-2383 injected intradermally in two doses was the only treatment which generated a titer against any of the antigens used in the IHA test. A titer of 40 was observed when P-2383 SR antigen was utilized to sensitize SRBC. The titer observed with the heat-extracted capsule of P-1062 was 10 both before and after injection of viable P-2383 intradermally. The titers against all other antigens utilized in the IHA test were zero.

Injection of Freund's complete adjuvant followed 2 months later by intravenous injection of heat-killed P-2383 mixed with 10 mg of ground Mycobacterium tuberculosis H-37RA failed to induce detectable levels of antibody.

Freeze-thawed P-2383 incorporated into Freund's complete adjuvant and injected subcutaneously in one dose failed to generate any demonstrable antibody.

Antibody titers produced in group 2 cattle

Table 4 presents the results of the indirect hemagglutination tests for the cattle in Group 2. Pre-immunization and highest post-immunization titers for each calf are presented for each antigen. Intradermal injection of viable Pasteurella multocida P-2383, P-2383 crude ribosomes and P-2383 RNA-protein antigen generated increased antibody titers in the majority of cattle to four of the five antigens used in the IHA test. Titers against P-2383 crude polysaccharide were generated in only four of ten calves. The maximum response to this antigen was a titer of 10.

Mean serum indirect hemagglutination titers of cattle in group 2

The mean indirect hemagglutination titers for cattle injected intradermally with viable Pasteurella multocida P-2383, P-2383 crude ribosomes (CR) or P-2383 RNA-protein are presented in Table 5. Sheep red blood cells (SRBC) were sensitized with P-2383 heat-extracted capsule, P-1062 heat-extracted capsule, P-2383 SR antigen, P-2383 phenol-extracted endotoxin, or P-2383 crude polysaccharide. Titers were read as the inverse of the highest dilution of serum giving positive agglutination. The highest titer observed prior to immunization is compared with the highest titer seen within 30 days following immunization. The calves were divided into three treatment groups based on the antigen injected. The mean pre-immunization titer for each group is compared to the mean highest post-injection titer for each group.

Table 4. Comparison of indirect hemagglutination titers before and after intradermal injection of viable Pasteurella multocida P-2383, crude ribosomes from P-2383 or RNA-protein from P-2383. Antigens used to sensitize sheep red blood cells were P-2383 heat-extracted capsule, P-2383 SR, P-1062 heat-extracted capsule, P-2383 phenol-extracted endotoxin or P-2383 crude polysaccharide. Titers are given as the inverse of the highest dilution of serum showing positive agglutination. The highest titer prior to antigen injection is compared to the highest titer after antigen injection for each animal. All sera were obtained prior to 30 days post-injection.

		TEST ANTIGEN									
		P-2383 heat-extracted capsule		P-2383 SR		P-1062 heat-extracted capsule		P-2383 phenol-extracted endotoxin		P-2383 crude polysaccharide	
Antigen injected intradermally	Calf #	Pre-I[a] Titer	Post-I[b] Titer	Pre-I Titer	Post-I Titer	Pre-I Titer	Post-I Titer	Pre-I Titer	Post-I Titer	Pre-I Titer	Post-I Titer
	3	0	0	0	40	10	10	0	0	0	0
	55	0	40	0	40	0	80	0	40	0	0
Viable	57	0	80	0	40	20	320	20	40	0	10
P-2383	61	10	80	10	80	20	160	10	80	0	0
	72	0	20	0	0	0	80	0	40	0	10
P-2383	56	10	80	10	80	20	320	10	40	0	0
crude	62	10	20	10	20	20	40	0	20	0	0
ribosomes	67	10	20	10	10	10	10	0	80	0	10
P-2383	64	10	20	10	20	10	40	0	20	0	0
RNA-	68	10	40	0	80	20	20	10	80	0	10
protein	71	10	20	10	20	0	10	10	40	0	0

[a]Highest titer observed prior to injection of a calf with the antigen indicated.

[b]Highest titer observed following antigen injection.

Table 5. Mean indirect hemagglutination titers of sera from calves immunized intradermally with viable Pasteurella multocida P-2383, crude ribosomes (CR) from P-2383, or RNA-protein from P-2383. Sheep red blood cells were sensitized with P-2383 heat-extracted capsule, P-1062 heat-extracted capsule, P-2383 (SR), P-2383 phenol-extracted endotoxin or P-2383 crude polysaccharide. Mean pre-immunization serum titers are compared to the highest mean post-immunization serum titers seen within 30 days following immunization. Calves are grouped by the antigen with which they were immunized. N equals the number of calves in each group.

			TEST ANTIGEN				
			Antigen Used to Sensitize SRBC				
Antigen Injected	No. calves	Serum	P-2383 heat-extracted capsule	P-1062 heat-extracted capsule	P-2383 SR	P-2383 phenol-extracted endotoxin	P-2383 crude polysaccharide
Viable P-2383	4	pre-injection	2	10	2	6	0
		post-injection	44	128	32	40	4
Crude Ribosomes	3	pre-injection	10	17	10	3	0
		post injection	40	123	37	47	3
RNA-Protein	3	pre-injection	10	10	7	7	0
		post-injection	27	23	47	47	3

Four calves (n = 4) received viable whole-broth cultures of P-2383 intradermally in two doses. The mean post-injection titers were: 1) 44 when P-2383 heat-extracted capsule was used to sensitize SRBC, 2) 128 against P-1062 heat-extracted capsule, 3) 32 against P-2383 SR antigen, 4) 40 against P-2383 phenol-extracted endotoxin and 5) 4 against P-2383 crude polysaccharide.

The intradermal injection of P-2383 crude ribosomes (CR) generated IHA antibody titers of 1) 40 against P-2383 heat-extracted capsule, 2) 123 against P-1062 heat-extracted capsule, 3) 37 against P-2383 antigen, 4) 47 against P-2383 phenol-extracted endotoxin, and 5) 3 against P-2383 crude polysaccharide. Three calves received the CR antigen intradermally in 2 doses.

The intradermal injection of P-2383 RNA-protein antigen generated mean IHA antibody titers of: 1) 27 against P-2383 heat-extracted capsule, 2) 23 against P-1062 heat-extracted capsule, 3) 47 against P-2383 SR antigen, 4) 47 against P-2383 phenol-extracted endotoxin, and 5) 3 against P-2383 crude polysaccharide. Three calves were injected intradermally with the P-2383 RNA-protein antigen in two doses.

Statistical comparison of mean indirect hemagglutination titers of cattle in Group 2

Table 6 presents a comparison of the mean IHA titers observed before and after intradermal injection of viable Pasteurella multocida P-2383, crude ribosomes (CR) from P-2383, or RNA-protein antigen from

Table 6. Comparison of mean pre-injection indirect hemagglutination titers of all cattle in Group 2 to the highest mean post-injection titers of Group 2 cattle injected with viable Pasteurella multocida P-2383, crude ribosomes from P-2383 and RNA protein from P-2383. Antigens used to sensitize sheep red blood cells were P-2383 heat-extracted capsule, P-1062 heat-extracted capsule, P-2383 SR antigen, P-2383 phenol-extracted endotoxin, or P-2383 crude polysaccharide. Titers are given as the mean ± Std error of the mean. N equals the number of individual animal titers. The two highest post-injection titers observed with each animal were used to compute the mean titer. All titers of 10 or less were assigned a value of 10. Standard error of the mean and level of significance (P) were computed on the $\log_{10}$ value of the titers. Level of significance was computed by the analysis of variance procedure. * = < 0.05; ** = P < 0.01.

Test antigen	Mean pre-injection titer of all cattle in group 2 ± Std error of the mean n = 10	Mean post-injection titer of cattle injected with viable P-2383 ± Std error of the mean n = 8	Mean post-injection titer of cattle injected with crude ribosomes ± Std error of the mean n = 6	Mean post-injection titer of cattle injected with RNA-protein ± Std error of the mean n = 6
P-2383 heat-extracted capsule	10 ± 0	39 ± 10**	28 ± 10*	27 ± 4**
P-1062 heat-extracted capsule	15 ± 2	121 ± 33**	76 ± 49	18 ± 5
P-2383 SR	10 ± 0	38 ± 10**	26 ± 11	32 ± 10*
P-2383 phenol-extracted endotoxin	11 ± 1	38 ± 7**	30 ± 11*	38 ± 10**
P-2383 crude polysaccharide	10 ± 0	10 ± 0	10 ± 0	10 ± 0

P-2383. The antigens used to sensitize sheep red blood cells (SRBC) were P-2383 heat-extracted capsule, P-1062 heat-extracted capsule, P-2383 SR antigen, P-2383 phenol-extracted endotoxin or P-2383 crude polysaccharide. The two highest titers following injection of the antigen were used to compute the mean titer. Cattle are grouped by the antigen which they received. Titers are given as the mean plus or minus the standard error of the mean. For statistical evaluation, titers of 10 or less were assigned a value of 10. Standard error of the mean and significance levels (P) were computed on the basis of the $\log_{10}$ of the titers by the analysis of variance procedure.

Viable Pasteurella multocida P-2383 injected intradermally in two doses to four cattle generated a highly significant ($P < 0.01$) increase in titer against all antigens except P-2383 crude polysaccharide.

Crude ribosomes (CR) injected intradermally in two doses to 3 cattle generated a significant ($P < 0.05$) antibody response to the P-2383 heat-extracted capsule and to the P-2383 phenol-extracted endotoxin. The mean titer against P-1062 heat extracted capsule was 76 but this titer proved not to be a statistically significant increase. A significant increase in mean antibody titer against the P-2383 crude polysaccharide was not observed.

The P-2383 RNA-protein antigen injected intradermally in two doses stimulated a highly significant ($P < 0.001$) increase in mean antibody titer against the P-2383 heat-extracted capsule and P-2383

phenol-extracted endotoxin. A significant increase in mean antibody titer was observed for the P-2383 SR antigen. The titers against P-1062 heat-extracted capsule and P-2383 crude polysaccharide were not significantly increased.

Gel diffusion precipitation

Photographs of the gel diffusion precipitation plates are presented in Figures 1-6. An attempt has been made to assign a number to each precipitin line to simplify the presentation of results. The presence or absence of precipitin lines in the photographs was markedly dependent upon the time photographs were taken following placement of antigens and antisera in the wells. Thus, some precipitin lines were observed early only to be obscured later by nonspecific precipitation. This nonspecific precipitation was observed as a clouding of the agar around the antigen wells and varied markedly between antigen preparations. Other precipitin lines were slow to develop and could not be visualized until 72-96 hours.

Figures 1 and 2 are photographs of gel diffusion precipitin plates in which antiserum produced by the injection of viable P-1062 was placed in the center wells. In Figure 1, starting at the top and moving clockwise, the antigens in the outer wells are crude ribosomes from P-2383 (CR), supernatant from the P-2383 ribosomes (SR), RNA-protein from P-2383 (RP), heat-extracted capsule of P-2383 (HE-2383), heat-extracted capsule of P-1062 (HE-1062), and phenol-

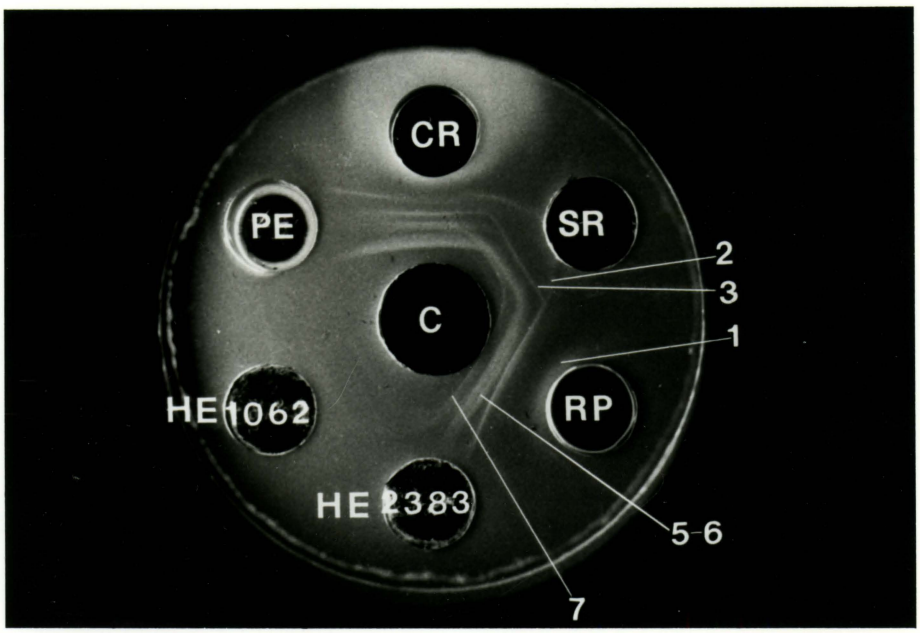

Figure 1. Gel diffusion precipitation analysis of the antigens of Pasteurella multocida. The center well contained serum (C) produced by intravenous injection of viable P-1062. The antigens in the outer wells were 1) Crude robosomes from P-2383 (CR), 2) Supernatant from the crude ribosomes (SR), 3) RNA-protein from P-2383 (RP), 4) Heat-extracted capsule of P-2383 (HE-2383), 5) Heat-extracted capsule of P-1062 (HE-1062) and 6) Phenol-extracted lipopolysaccharide from P-2383 (PE).

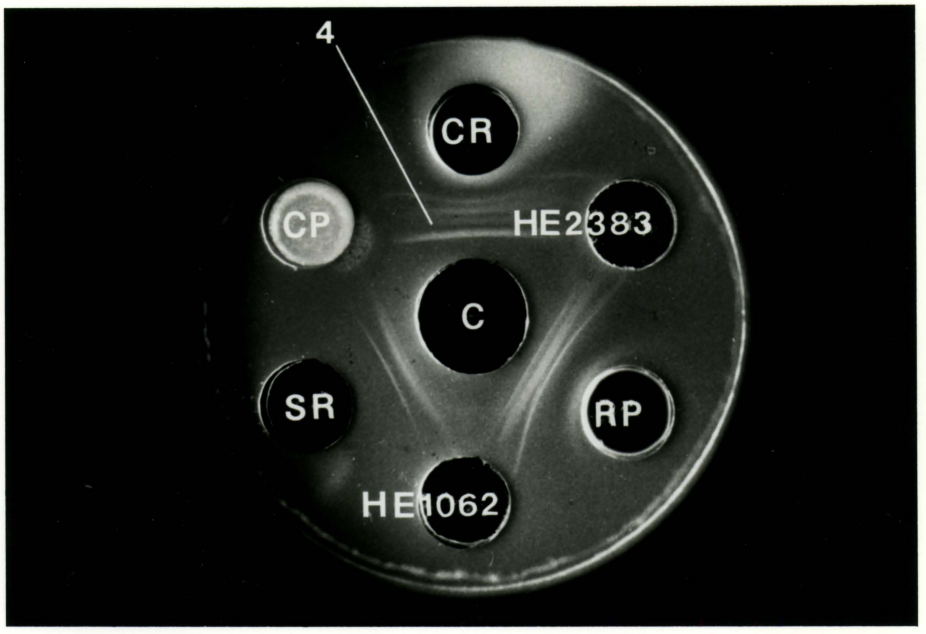

Figure 2. Gel diffusion precipitation analysis of the antigens of Pasteurella multocida. The center well contained serum (C) produced by multiple intravenous injection of viable P-1062. The antigens in the outer wells were 1) Crude ribosomes from P-2383 (CR), 2) Heat-extracted capsule from P-2383 (HE-2383), 3) RNA-protein from P-2383 (RP), 4) Heat-extracted capsule from P-1062 (HE-1062), 5) Supernatant from crude ribosomes (SR), and 6) Phenol-extracted lipopolysaccharide from P-2383 (PE).

extracted lipopolysaccharide from P-2383 (PE). In Figure 2, starting at the top and moving clockwise the antigens in the outer wells are CR, HE-2383, RP, HE-1062, SR and crude polysaccharide from P-2383 (CP).

The antiserum produced by injection of viable P-1062 was observed to give the clearest pattern of precipitin lines on gel diffusion precipitation. In Figure 1, a precipitin line appears near the antigen well of the RP preparation which is designated line number 1. This line was present in the CR fraction and, because of subsequent nonspecific clouding of the gel could only be observed within 36 hr. after the antigen was placed in the well. Antigen #1 was present in the heat-extracted capsules of P-2383 and P-1062 and in the phenol extract of P-2383 (PE) although the precipitin lines did not photograph well. Line #1 was the predominant precipitin line observed with the phenol-extracted lipopolysaccharide and thus in all likelihood represents the endotoxin component of P-2383. Direct observation of the gel diffusion precipitin plates occasionally revealed the presence of two precipitin lines within line #7 of the PE fraction.

Figure 1 illustrates the presence of two precipitin lines in the RP preparation designated 2 and 3. These lines are essentially superimposed but can be observed as two distinct entities one of which (3) gives a line of identity with a line in the SR and CR preparations and one (2) which is contained only in the CR and RP preparations.

A distinct difference is observed in the appearance of antigens 2 and 3 between the CR and RP preparations. The two precipitin lines are well-separated in the CR preparation and are superimposed in the RP fraction. This apparently represents different concentrations of antigen 2 in these fractions. Precipitin lines 2 and 3 were not observed in any of the other bacterial fractions. Another interesting point is demonstrated in Figure 2. Precipitin line 2 as it appears in the CR fraction is composed of two distinct antigens. The second component of this precipitin line is observed in the upper right of the plate. If one now observes the same area in Figure 1, these two components can be visualized albeit not as distinctly as in Figure 2.

Precipitin line 4 can be observed clearly in Figure 2. The antigen represented by this line is apparently unique to the CR fraction although this distinction is not readily evident in Figure 1. The possibility exists that this antigen may also be present in the RP preparation at a different concentration. Also, there is some indication that two distinct antigens may be represented in this precipitin line.

Antigens 5 and 6 are observed as two precipitin lines in the RP fraction in Figure 1. The distinction between these two components is blurred in the CR fraction, although two precipitin lines can be observed. It appears that only one of these antigens is represented in the SR fraction, however, whether antigen 5 or 6 is the antigen present is unclear. At least one of these antigens is also present in the heat-extracted capsules of P-2383 and P-1062 and possibly in the

crude polysaccharide of P-2383. This line was extremely weak and did not photograph well.

Antigen 7 is clearly visualized in the RP fraction in Figure 2 near the antiserum well. This component was also readily observed in the SR fraction. The CR fraction apparently contained a lesser amount of this antigen as can be observed in Figure 1. This antigen was not observed in the other bacterial fractions.

Figures 3 and 4 are photographs of gel diffusion precipitin plates in which antiserum produced by the injection of viable P-2383 was placed in the center well. The antigens are arranged in the outer wells in a fashion identical to that in Figures 1 and 2, respectively. Figure 3 was photographed at an early stage following addition of the antigens and antiserum to the respective wells, thus the precipitin lines are not as distinct.

In the CR fraction, precipitin lines 1 through 6 were observed although they photographed poorly in Figures 3 and 4. A major problem observed with the homologous P-2383 antiserum was the presence of a rather diffuse precipitation reaction. This appeared only in the CR, SR, RP and phenol-extracted antigens. Within this diffuse precipitation band, separate precipitin lines were discerned in the CR, SR and RP fractions. Thus, it appears that a multiplicity of antigens is present within these fractions as represented by the diffuse precipitation bands.

The presence of a portion of either antigen 5 or 6 in the P-1062 heat-extrated capsule is demonstrated in Figure 4. This reaction

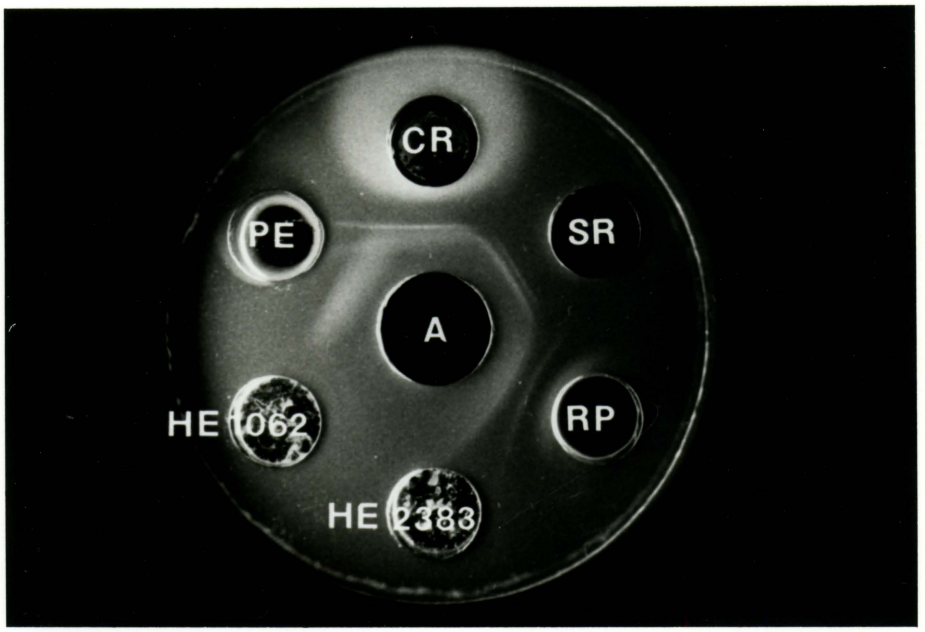

Figure 3. Gel diffusion precipitation analysis of the antigens of Pasteurella multocida. The center well contained serum (A) produced by multiple intravenous injection of viable P-2383. The antigens in the outer wells were: 1) Crude ribosomes from P-2383 (CR), 2) Supernatant from the ribosomes (SR), 3) RNA-protein from P-2383 (RP), 4) Heat-extracted capsule of P-2383 (HE-2383), 5) Heat-extracted capsule of P-1062 (HE-1062), and 6) Phenol-extracted lipopolysaccharide from P-2383 (PE).

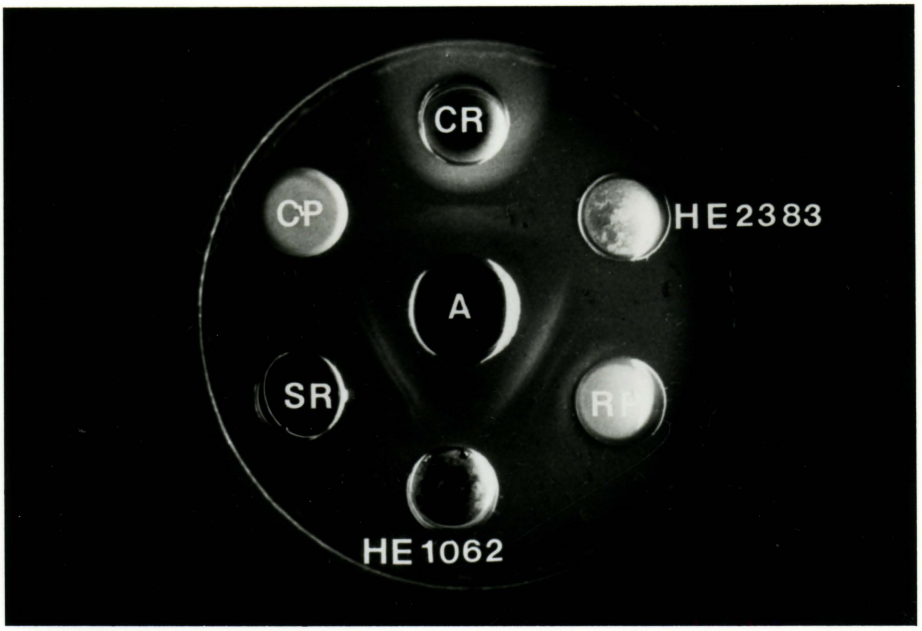

Figure 4. Gel diffusion precipitation analysis of the antigens of Pasteurella multocida. The center well contained serum (A) produced in cattle by multiple intravenous injection of viable P-2383. The antigens added to the outer wells were 1) Crude ribosomes from P-2383 (CR), 2) Heat-extracted capsule from P-2383 (HE-2383), 3) RNA-protein from P-2383 (RP), 4) Heat-extracted capsule from P-1062 (HE-1062), 5) Supernatant from crude ribosomes (SR), and 6) Phenol-extracted lipopolysaccharide from P-2383 (PE).

was relatively weak, however.

Precipitin lines 1, 2-3, and 5-6 were observed to form in the RP preparation against the homologous antiserum produced by injection of viable P-2383. The precipitin line representing antigen 7 is in all probability obscured in the diffuse precipitin band. Precipitin lines 3 and 5-6 were observed in the SR fraction. Again, precipitin line 7 may be present but is obscured.

Figures 5 and 6 are photographs of gel diffusion precipitin plates in which bovine antiserum to heat-killed P-2383 was placed in the center wells. The antigens are arranged identically to Figures 1 and 2, respectively. Figure 5 was photographed at an early stage following addition of the respective antiserum and antigens to the proper wells. Figure 6 is a photograph taken at a later stage. Precipitin lines 1-6 were observed to form in the CR fraction against this antiserum. The diffuse precipitin lines observed to form against the serum produced by injection of viable P-2383 were not as pronounced as with the serum produced by injection of heat-killed organisms The patterns of precipitin lines observed with the RP fraction indicated the presence of antigens 1, 2-3, 5-6 and possibly 7. The SR fraction demonstrated antigens 3, 5-6 and 7. In Figure 6 antigen 7 is observed to be more diffuse than it appeared in Figure 1. The phenol-extracted lipopolysaccharide in Figure 5 demonstrates a diffuse band similar in position to that which appears in Figure 3. The line of partial identity observed in Figure 3 between this band and precipitin lines 5-6 is not as readily apparent in Figure 3.

Weak precipitin lines were formed against the heat-extracted

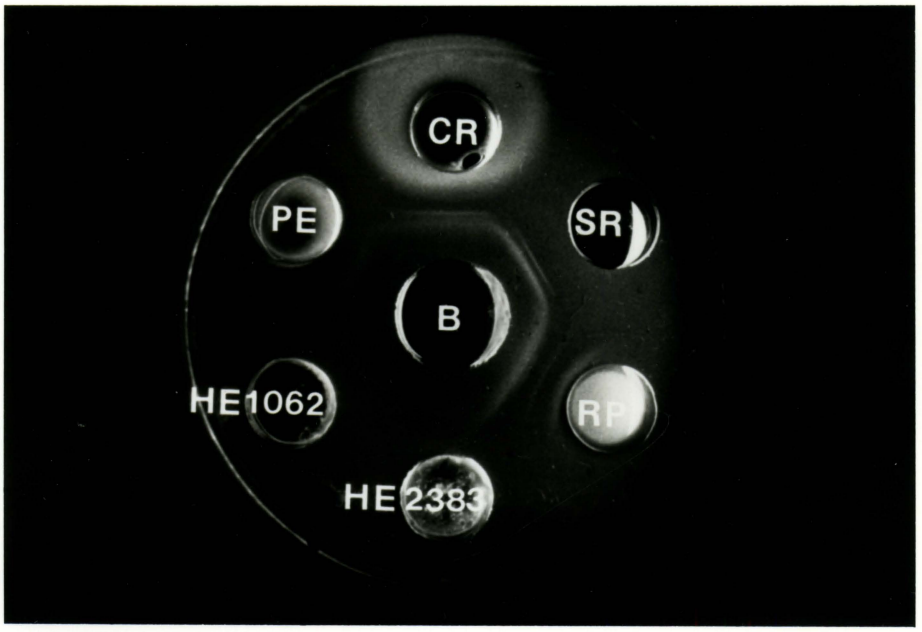

Figure 5. Gel diffusion precipitation analysis of the antigens of Pasteurella multocida. The center well (B) contained serum produced by multiple subcutaneous injection of heat-killed P-2383 incorporated in aluminum hydroxide gel. The antigens in the outer wells were: 1) Crude ribosomes from P-2383 (CR), 2) Supernatant from the ribosomes (SR), 3) RNA-protein from P-2383 (RP), 4) Heat-extracted capsule of P-2383 (HE-2383), 5) Heat-extracted capsule of P-1062 (HE-1062), and 6) Phenol-extracted lipopolysaccharide from P-2383 (PE).

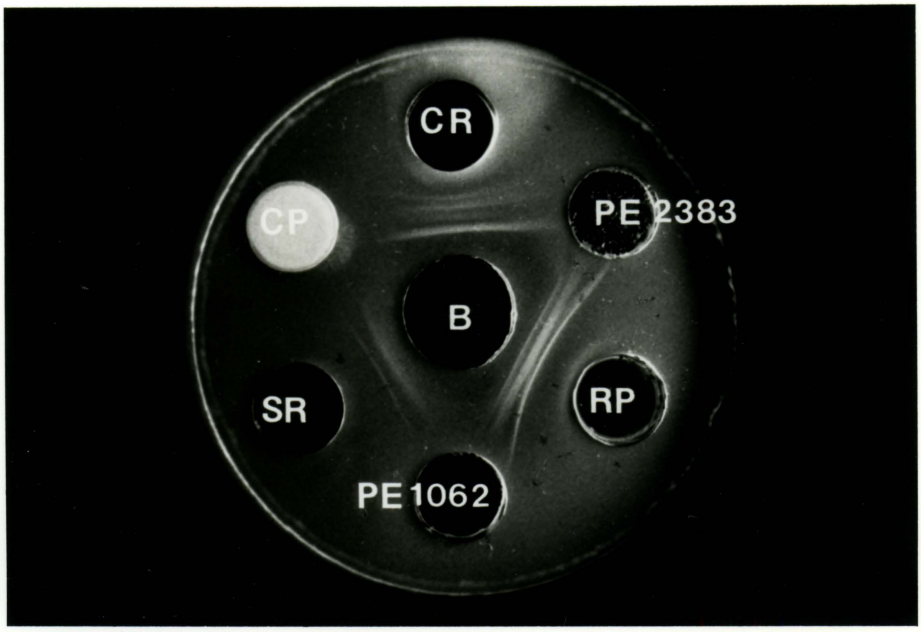

Figure 6. Gel diffusion precipitation analysis of the antigens of Pasteurella multocida. The center well contained serum (B) produced in cattle by multiple subcutaneous injections of heat-killed P-2383 incorporated in aluminum hydroxide gel. The antigens in the outer wells were: 1) Crude ribosomes from P-2383 (CR), 2) Heat-extracted capsule from P-2383 (HE-2383), 3) RNA-protein from P-2383 (RP), 4) Heat-extracted capsule from P-1062 (HE-1062), 5) Supernatant from crude ribosomes (SR), and 6) Phenol-extracted lipopolysaccharide from P-2383 (PE).

capsules of P-2383 and P-1062 and the crude polysaccharide of P-2383. These weak precipitin lines were discerned with difficulty on the gel diffusion plates. It appeared that all three preparations contained antigen 1. The heat-extracted capsular preparations contained antigens which gave precipitin bands in the area of lines 3 and 5-6. None of the precipitin lines observed with these three fractions was readily apparent and as such did not photograph well.

Immunoelectrophoresis

Diagrams of the immunoelectrophoretic analyses of the antigens of P. multocida are presented in Figures 7-9. The antigen added to each of the wells is indicated to the left of the well. Antigens examined were P-2383 crude ribosomes (CR), P-2383 RNA-protein (RP), P-2383 supernatant from the ribosomes (SR), P-1062 heat-extracted capsule (HE-1062), P-2383 heat-extracted capsule (HE-2383) and P-2383 phenol-extracted lipopolysaccharide (PE). Antigens were applied to the wells of the immunoelectrophoresis plates and electrophoresed as described in materials and methods. Antisera were then added to each of the troughs. Figures 7, 8, and 9 are diagrams of the immunoprecipitin bands observed with antiserum produced by immunization of cattle with viable P-2383, heat-killed P-2383, and viable P-1062 respectively. In an attempt to simplify discussion, each of the precipitation bands has been assigned a letter.

Figure 7 represents the immunoelectrophoretograms observed with antiserum produced by multiple intravenous injection of viable P-2383.

A complex of antigen labeled A, B and C was observed in both the CR and RP fractions. One of these antigens appeared to be absent in the SR fraction. This antigen was thus probably identical to antigen 2 as observed in the gel diffusion precipitation test. This missing antigen appears to be antigen A. The A, B, C complex was not observed in the immunoelectrophoretograms of the heat-extracted antigens.

Antigen D appeared in the RP and SR fractions but not in the others. This antigen may be identical to antigen 7 as observed in the gel diffusion precipitation analyses. Antigen 7 appeared in the CR fraction on gel diffusion precipitation; however, its position indicates a lower concentration was present in the CR fraction. Thus it may not appear in the immunoelectrophoretograms.

Antigen E in Figure 7 is unique to the RP fraction of P-2383. The relationship of this antigen to those observed on gel diffusion precipitation could not be ascertained.

Antigen F or components of it were observed in all of the antigenic fractions analyzed on immunoelectrophoresis except the SR fraction. This antigen probably represents antigen 1 as observed in gel diffusion precipitation. It contains few charged groups at pH 8.2 as indicated by its poor movement in the immunoelectrophoretogram and diffuses through the agarose gel very poorly. These characteristics are consistent with a high molecular weight lipopolysaccharide moiety. Within the area defined by the F antigen, the presence of two separate components was observed in the CR fraction.

Figure 7 illustrates the presence of a long diffuse trail of

heterogeneous material which formed a precipitate on the immunoelectrophoretogram. This precipitate was labeled G. It probably represents the diffuse precipitation observed on some of the gel diffusion precipitin plates. The G component(s) was most pronounced in the CR fraction and less so in the SR and heat-extracted capsules of P-2383 and P-1062. This diffuse band did not appear in the RP or phenol-extracted lipopolysaccharide preparations.

Figure 8 represents the immunoelectrophoretograms of the antigens of P. multocida as they appeared when antiserum produced by immunization of cattle with heat-killed P-2383 was placed in the troughs. The appearance of these immunoelectrophoretogram was essentially similar to that diagrammed in Figure 7 with one exception: an additional antigen (H) appears in the CR and RP fractions. This antigen was observed to be relatively weak. The presence of it in Figure 8 and absence of it in Figure 7 either represents a clarification of the lines because of a lack of a hazy precipitate or a quantitative difference in the amount of specific antibody present in each of the homologous antisera.

Figure 9 illustrates the electrophoretogram of the antigens of P. multocida observed when antiserum generated by the intravenous inoculation of cattle with viable P-1062 was placed in the troughs of the immunoelectrophoresis plates. No major differences existed between this immunoelectrophoretogram and those of the homologous sera. The E antigen was absent from the RP preparation and the H antigen was not readily apparent in the same fraction.

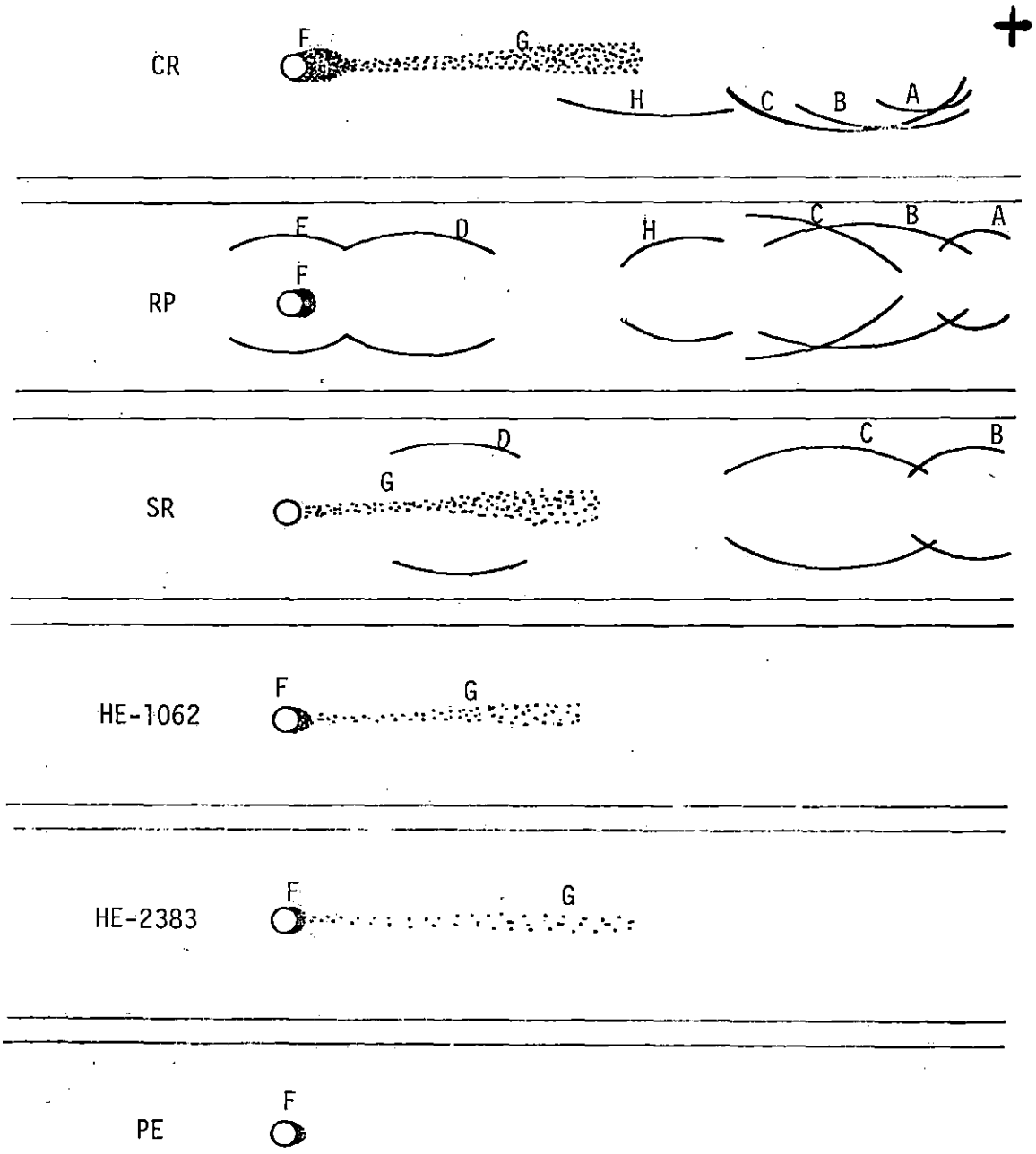

Figure 7. Diagrammatic illustration of immunoelectrophoretograms analyzing the antigen content of several fractions isolated from Pasteurella multocida. Antigens added to the wells and electrophoresed were: (1) P-2383 CR antigen (CR), (2) P-2383 RNA-protein antigen (RP), (3) P-2383 SR antigen (SR), (4) P-1062 heat-extracted capsule (HE-1062), (5) P-2383 heat extracted capsule (HE-2383), and (6) P-2383 phenol-extracted lipopolysaccharide (PE). The antiserum added to the troughs was produced in cattle by multiple subcutaneous injections of heat-killed P-2383 incorporated in aluminum hydroxide gel.

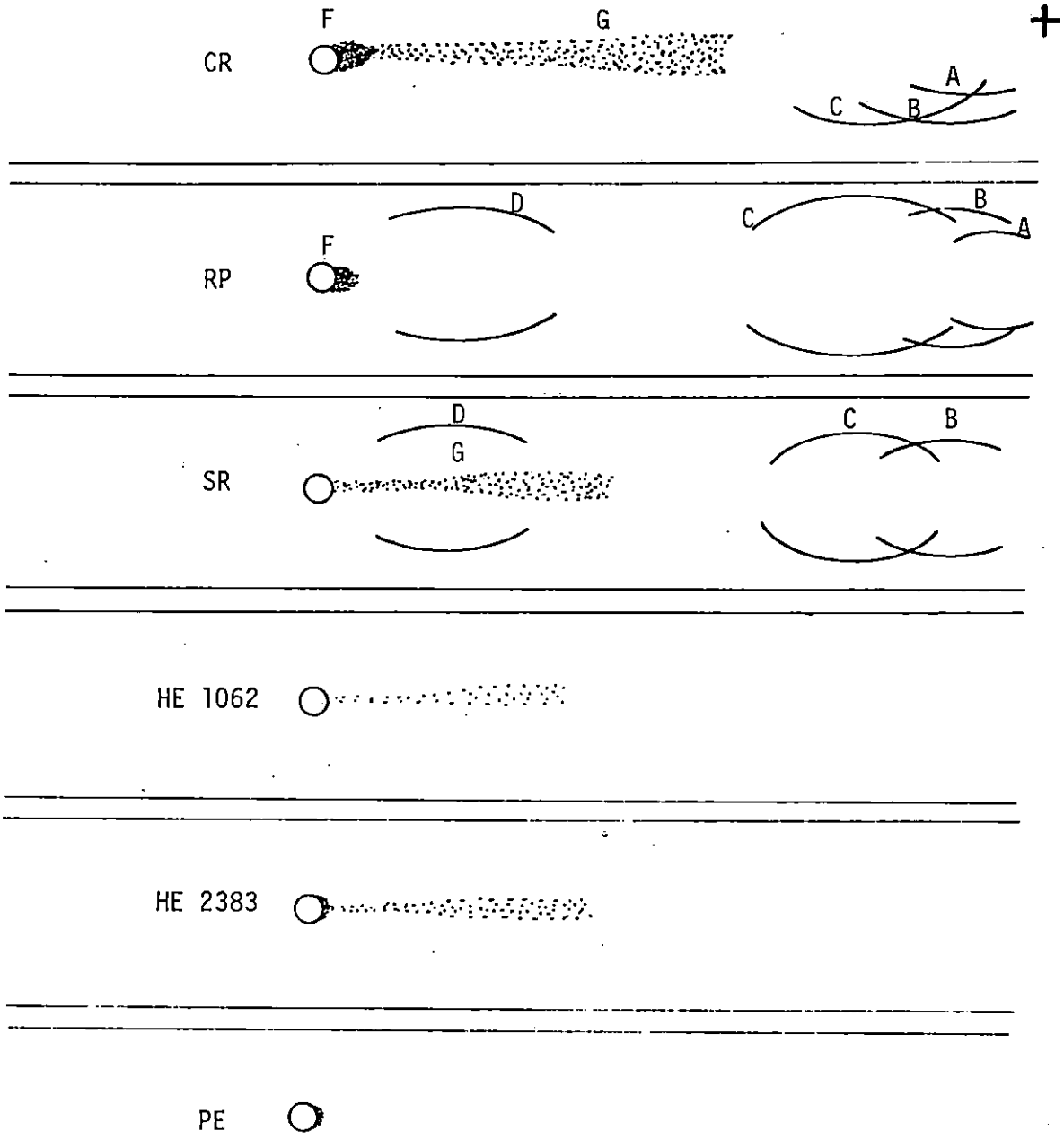

Figure 8. Diagrammatic illustration of immunoelectrophoretograms analyzing the antigen content of several fractions isolated from Pasteurella multocida. Antigens added to the wells and electrophoresed were: (1) P-2383 CR antigen (CR), (2) P-2383 RNA-protein antigen (RP), (3) P-2383 SR antigen (SR), (4) P-1062 heat extracted capsule (HE 1062), (5) P-2383 heat extracted capsule (HE 2383), and (6) P-2383 phenol-extracted lipopolysaccharide (PE). The antiserum added to the troughs was produced in cattle by multiple intravenous inoculations of viable P-1062.

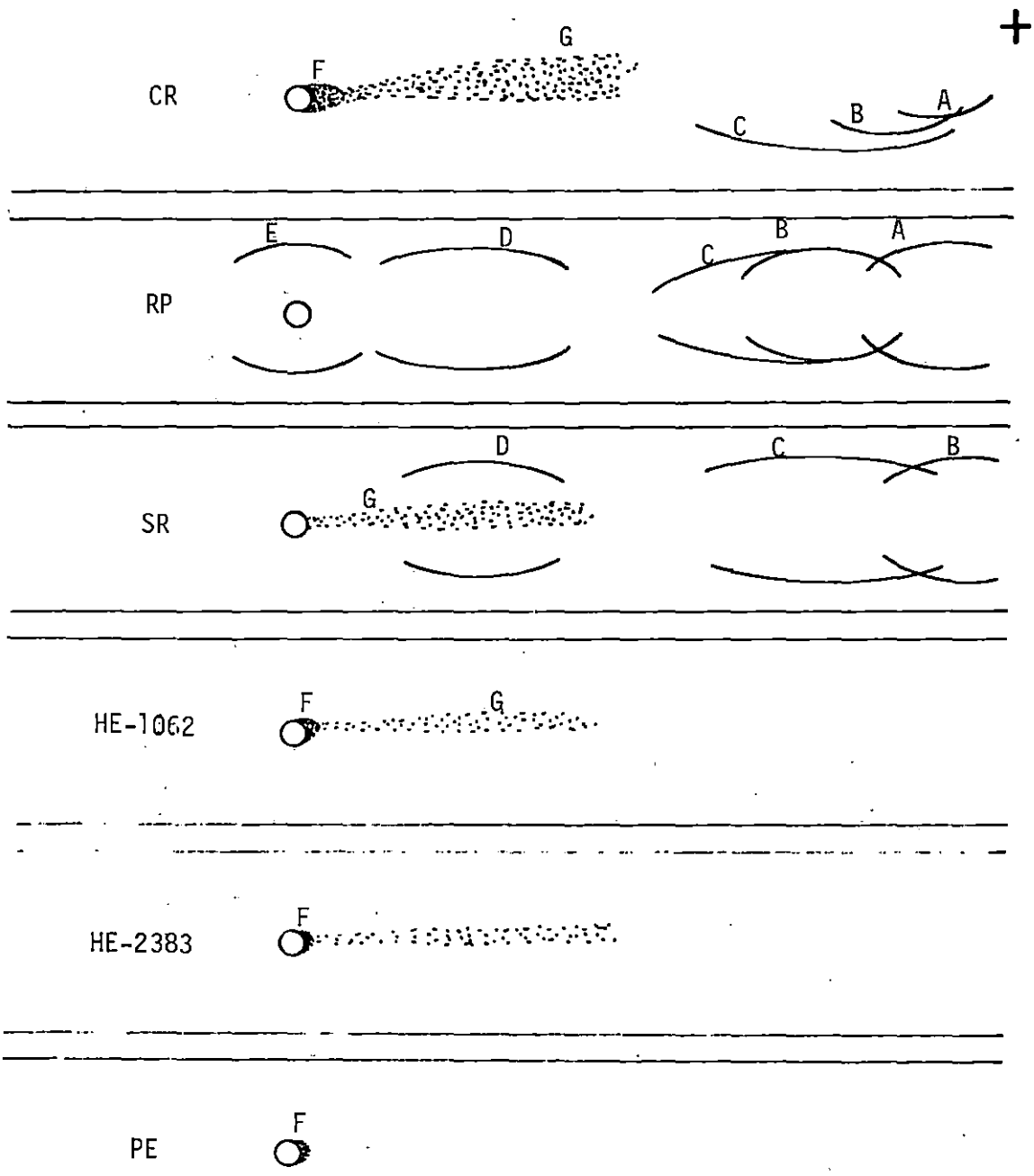

Figure 9. Diagrammatic illustration of immunoelectrophoretograms analyzing the antigen content of several fractions isolated from Pasteurella multocida. Antigens added to the wells and electrophoresed were: (1) P-2383 CR antigen (CR), (2) P-2383 RNA-protein antigen (RP), (3) P-2383 SR antigen (SR), (4) P-1062 heat extracted capsule (HE-1062), (5) P-2383 heat extracted capsule (HE-2383) and (6) P-2383 phenol-extracted lipopolysaccharide (PE). The antiserum added to the troughs was produced in cattle by multiple intravenous inoculations of viable P-2383.

Lymphocyte blastogenesis

Selected antigens were tested in lymphocyte cultures to evaluate their toxicity and blastogenic activity. It became evident from preliminary experimentation that a screening procedure to limit the number of antigens and the number of concentrations of antigens was essential. An outbreak of pneumonia from which a Type A Pasteurella multocida was isolated occurred in a group of 6-7 month old Holstein-Fresian dairy calves belonging to Iowa State University. Peripheral blood lymphocytes were isolated from these animals and used to screen the various antigens. If antigens were found to routinely suppress lymphocyte blastogenesis below a stimulation index of 0.8, they were diluted further. Concentrations observed to give the highest stimulation indices for a given antigen were subsequently used in the later Group 1 investigations. Antigen preparations were added to the investigation or deleted from it as indicated by lack of response.

The results of the lymphocyte blastogenesis investigation for calves 2, 3, 5, and 7 (Group 1) are presented in Tables 7 and 8.

The ability of P. multocida P-2383 crude polysaccharide, P-2383 phenol-extracted endotoxin, P-2383 saline-extracted capsule, P-2383 free endotoxin, P-2383 crude ribosomes (Cr) and P-2383 RNA-protein to stimulate a blastogenic response in lymphocyte cultures of calves in Group 1 is presented in Table 7. The amount of each antigen added to each well of a microtiter plate is given. The values listed are mean stimulation indices plus or minus the standard error of the mean. The number of observations in each group (n) is given. Mean stimulation indices are reported for the control group which includes all

observations from calf #2 plus all observations on calves 3, 5 and 7 prior to antigen injection. Calf #3 received viable P-2383 intradermally in two doses. Calf #5 received a subcutaneous injection of Freund's complete adjuvant followed two months later by a single intravenous inoculation of heat-killed P-2383 mixed with 10 mg of ground Mycobacterium tuberculosis H-37RA. Calf #7 received freeze-thawed P-2383 incorporated in Freund's complete adjuvant in a single subcutaneous dose. Significance levels (P) were computed on the $\log_{10}$ value of the stimulation indices by the analysis of variance procedure.

Viable P-2383 injected intradermally was the only antigen to stimulate a highly significant ($P < 0.01$) blastogenic response in the bovine lymphocyte cultures. The stimulation index for the 25 µg level (protein basis, BR) of the P-2383 RNA-protein antigen was 4.2 ± 0.74. The 50 µg level of RNA-protein gave a stimulation index of 3.3 ± 1.25. The 25 µg level of the P-2383 CR antigen produced a mean stimulation index of 4.2 ± 0.49 while the 50 µg level produced a mean stimulation index of 2.4 ± 0.78. By analysis of variance, the blastogenic response to the 50 µg level of RNA protein, and 25 µg and 50 µg levels of CR antigen were not significant.

Injection of Freund's complete adjuvant followed by intravenous administration of heat-killed P-2383 plus ground M. tuberculosis did not stimulate a significant blastogenic response in bovine lymphocyte cultures.

Administration of freeze-thawed P-2383 incorporated in Freund's complete adjuvant did not stimulate a significant blastogenic response to any of the antigens listed in Table 7.

Table 7. Ability of P. multocida P-2383 crude polysaccharide, P-2383 phenol-extracted endotoxin, P-2383 saline-extracted capsule, P-2383 free endotoxin, P-2383 crude ribosomes and P-2383 RNA-protein to stimulate a blastogenic response in cultures of lymphocytes from cattle in Group I. The antigens and the amounts added to each well of a microtiter plate are listed. The values given are mean stimulation indices plus or minus the standard error of the mean. N equals the number of observations in each group. Mean stimulation indices are given for the control group which includes all observations from calf #2 plus all observations on calves 3, 5 and 7 prior to antigen injection. Calf #3 received viable P-2383 intradermally in two doses. Calf #5 received a subcutaneous injection of Freund's complete adjuvant. This was followed 2 months later by an intravenous administration of heat-killed P-2383 mixed with ground Mycobacterium tuberculosis H-37RA. Calf #7 received freeze-thawed P-2383 incorporated into Freund's complete adjuvant. Significance levels (P) were calculated on the $\log_{10}$ of the stimulation indices by the analysis of variance procedure. ** = $P < 0.01$.

	n	20 g P-2383 crude poly-saccharide	18 g P-2383 phenol-extracted capsule	20 g P-2383 saline-extracted capsule	20 g P-2383 free endotoxin
Control	8	0.9 ± 0.25	1.2 ± 0.44	1.5 ± 0.44	1.2 ± 0.26
Calf #3	3	1.6 ± 0.77	0.5 ± 0.18	1.2 ± 0.25	0.8 ± 0.15
Calf #5	5	1.2 ± 0.36	0.7 ± 0.25	1.1 ± 0.31	1.3 ± 0.23
Calf #7	5	0.6 ± 0.07	0.4 ± 0.7	0.9 ± 0.08	1.1 ± 0.36

25 g P-2383 CR	50 g P-2383 CR	25 g P-2383 RNA-protein	50 g P-2383 RNA-protein
2.1 ± 0.67	0.9 ± 0.18	0.9 ± 0.29	1.2 ± 0.20
4.2 ± 0.49	2.4 ± 0.78	4.2 ± 0.74**	3.3 ± 1.25
2.2 ± 0.88	1.4 ± 0.56	2.1 ± 0.70	2.4 ± 0.96
1.6 ± 0.44	1.1 ± 0.29	1.9 ± 0.47	1.3 ± 0.56

The ability of the Pasteurella multocida P-2383 heat-extracted capsular material to stimulate a blastogenic response in cultures of bovine lymphocytes is presented in Table 8. The level of heat-extracted antigen added to each well of a microtiter plate is given. The mean stimulation indices plus or minus the standard error of the mean are listed for the control group and for calves 3, 5 and 7. The number of observations (n) for the control group includes all observations for calf #2 plus all observations on calves 3, 5, and 7 prior to antigen injection. Significance levels were computed on the $\log_{10}$ of the stimulation indices by the analysis of variance procedure.

Heat-extracted capsular antigen from P-2383 was unable to stimulate blastogenesis in lymphocyte cultures in any of the calves in Group 1. Levels of heat-extracted capsule added to each well of a microtiter plate ranged from 4 µg to 186 µg (dry weight). Amounts above 186 µg routinely resulted in a marked depression of lymphocyte blastogenesis and were discontinued in the early stages of the investigation.

Lymphocyte blastogenesis: Group 2 cattle

The results of the lymphocyte blastogenesis investigations on cattle in Group 2 are presented in Tables 9 and 10.

The abilities of Pasteurella multocida P-2383 saline extracted capsule, P-2383 phenol-extracted endotoxin, and trichloroacetic acid (TCA) extracted antigen to stimulate a blastogenic response in cultures of lymphocytes from calves in Group 2 are presented in Table 9.

Table 8. Blastogenic response of lymphocytes from Group I heat-extracted capsular antigen from P-2383. The level of heat-extracted antigen added to each well of a microtiter plate is listed. The mean stimulation indices plus or minus the standard error of the mean are given for the control group and calves 3, 5 and 7. N equals the number of observations in the control group and for each calf. The mean stimulation indices for the control group include all observations from calf #2 plus all observations on calves 3, 5 and 7 prior to antigen injection. Calf #2 acted as the negative control. Calf #3 received viable P-2383 intradermally in two doses. Calf #5 was injected subcutaneously with Freund's complete adjuvant. Two months later heat-killed P-2383 mixed with ground Mycobacterium H-37RA was administered intravenously. Calf #7 received one dose of freeze-thawed P-2383 incorporated into Freund's complete adjuvant. Significance levels (P) were calculated on the $\log_{10}$ of the stimulation indices for each observation by the analysis of variance procedure. * = $P < 0.05$. ** = $P < 0.01$.

	n	Level of heat-extracted capsule				
		186 µg	93 µg	37 µg	18 µg	4 µg
Control	8	1.2 ± 0.27	1.4 ± 0.35	1.5 ± 0.35	1.6 ± 0.35	1.3 ± 0.29
Calf #3	3	0.8 ± 0.05	1.1 ± 0.12	1.0 ± 0.13	0.7 ± 0.18	1.1 ± 0.36
Calf #5	5	1.3 ± 0.18	1.4 ± 0.32	1.3 ± 0.15	1.2 ± 0.23	1.5 ± 0.17
Calf #7	5	1.2 ± 0.18	0.9 ± 0.26	0.9 ± 0.26	0.8 ± 0.23	0.8 ± 0.23

Table 9. Ability of Pasteurella multocida P-2383 saline extracted capsule, P-2383 phenol-extracted endotoxin, and P-2383 trichloroacetic acid (TCA)-extracted antigen to stimulate a blastogenic response in cultures of bovine lymphocytes from Group 2 calves. The antigens and amounts added to each well of a microtiter plate are listed. The mean stimulation indices plus or minus the standard error of the mean are given for the control observations and for cattle receiving viable P-2383 intradermally, P-2383 crude ribosomes intradermally and P-2383 RNA-protein intradermally. Each group of animals receiving a particular antigen is divided into three periods on the basis of the number of days following injection of the antigen. N equals the number of observations in each group. The control group consists of all observations on all Group 2 cattle prior to injection of antigen. Level of significance (P) was computed on the $\log_{10}$ value of the stimulation indices by the analysis of variance procedure. * = $P < 0.05$. ** = $P < 0.01$.

	Days post injection	25 µg saline-extracted capsule	50 µg saline-extracted capsule	9 µg phenol-extracted endotoxin	18 µg phenol-extracted endotoxin	1:500 TCA extract	1:1000 TCA-extract
Controls	0	0.8 ± 0.07	1.2 ± 0.15	1.3 ± 0.15	1.1 ± 0.14	0.9 ± 0.08	0.9 ± 0.12
n =		55	55	54	54	41	41
Viable P-2383	1-14 n = 8	2.3 ± 0.90**	2.8 ± 1.06	1.7 ± 0.48	3.0 ± 1.26	0.9 ± 0.13	0.7 ± 0.09
	15-20 n = 8	0.9 ± 0.7	1.5 ± 0.28	1.2 ± 0.16	1.9 ± 0.35	0.9 ± 0.08	0.8 ± 0.10
	> 20 n = 9	1.0 ± 0.10	1.6 ± 0.28	1.6 ± 0.28	2.2 ± 0.76	0.7 ± 0.11	0.7 ± 0.09
P-2383 crude ribosomes	1-14 n = 7	0.9 ± 0.14	1.8 ± 0.57	1.8 ± 0.64	2.4 ± 1.55	0.8 ± 0.11	0.7 ± 0.11
	15-20 n = 7	0.7 ± 0.9	1.2 ±	0.9 ± 0.40	1.0 ± 0.18	0.9 ± 0.23	0.8 ± 0.20
	> 20 n = 7	1.1 ± 0.32	1.9 ± 0.62	1.6 ± 0.28	1.1 ± 0.18	0.7 ± 0.19	0.7 ± 0.27

P-2383 RNA-protein	1-14 n = 8	0.9 ± 0.12	2.8 ± 1.18	1.9 ± 0.71	2.2 ± 1.25	0.8 ± 0.16	0.7 ± 0.20
	15-20 n = 8	0.7 ± 0.09	0.8 ± 0.13	1.1 ± 0.35	1.9 ± 0.79	0.8 ± 0.08	0.6 ± 0.10
	> 20 n = 7	1.2 ± 0.17*	1.8 ± 0.40*	2.0 ± 0.45	2.2 ± 0.67*	1.0 ± 0.18	0.8 ± 0.09

The antigens and amounts added to each well of a microtiter plate are stated. Values reported are mean stimulation indices plus or minus the standard error of the mean. Cattle were injected intradermally with either viable P-2383, P-2383 crude ribosomes (CR), or P-2383 RNA-protein. The mean stimulation indices were grouped into three time periods on the basis of the number of days following the last injection of antigen. The number of observations in each group (n) is given. Data in the control group consist of observations on Group 2 cattle prior to injection of antigen. The level of significance (P) was computed on the $\log_{10}$ value of the stimulation indices by the analysis of variance procedure.

Injection of viable P-2383 intradermally resulted in a highly significant ($P < 0.01$) stimulation of lymphocytes against the saline-extracted capsule of P-2383 at the 25 µg level during days 1-14. The mean stimulation index for this level of the antigen was 2.3 ± 0.90. The 50 µg level for this same time period had a mean stimulation index of 2.8 ± 1.05 but this did not reach significance.

P-2383 phenol extracted endotoxins gave a mean stimulation index at the 18 µg level of 3.0 ± 1.26 during days 1-14. This also did not reach significance.

The intradermal injection of P-2383 crude ribosomes did not result in any significant sensitization of bovine lymphocytes to saline-extracted capsule, phenol-extracted endotoxin or TCA-extracted endotoxin during any of the post-injection time periods. A mean stimulation index of 2.4 ± 1.55 was observed for the 18 µg level of

phenol-extracted endotoxin.

The intradermal injection of P-2383 RNA-protein resulted in significant ($P < 0.05$) sensitization of peripheral bovine lymphocytes to the saline-extracted capsule and phenol-extracted endotoxin during the time period from 20 days on. Both the 25 and 50 µg levels of the saline-extracted capsular antigen produced significant stimulation although the mean stimulation indices were only 1.2 ± 0.17 and 1.8 ± 0.40 respectively. The days 1-14 mean stimulation index for the 50 µg level of the same antigen produced a mean stimulation index of 2.8 ± 1.18 but this did not approach significance.

The phenol-extracted endotoxin produced mean stimulation indices for bovine lymphocyte cultures of 2.0 ± 0.45 and 2.2 ± 0.67 for the 9.0 and 18 µg levels. Only the latter was significant ($P < 0.05$).

The response of lymphocytes from cattle in Group II to Pasteurella multocida P-2383 crude ribosome (CR), P-2383 supernatant from the ribosomes (SR), and P-2383 RNA-protein to stimulate blastogenesis in cultures of lymphocytes from cattle in Group 2 is presented in Table 10. The antigens and amounts added to each well of a microtiter plate are given. The mean stimulation indices plus or minus the standard error of the mean are listed. Cattle in Group 2 received either viable P-2383 intradermally, P-2383 crude ribosomes intradermally or P-2383 RNA-protein intradermally. Animals are grouped on the basis of the antigen injected and each group is divided into three time periods on the basis of the time following antigen injection. The number of observations (n) in each time period is given. The

Table 10. Blastogenic response of lymphocytes from Group II cattle to Pasteurella multocida P-2383 crude ribosomes (CR), P-2383 supernatant from the ribosomes (SR), and P-2383 RNA-protein to stimulate blastogenesis in cultures of lymphocytes from cattle in Group II. The antigens and the amounts added to each well of a microtiter plate are reported. The mean stimulation indices plus or minus the standard error of the mean are given for the control observations and for cattle receiving viable P-2383 intradermally, P-2383 crude ribosomes intradermally, and P-2383 RNA-protein intradermally. Each group of animals receiving a particular antigen is divided into three time groups on the basis of the number of days following the injection of the antigens. N equals the number of observations in each group. The control group consists of all observations on Group II cattle prior to injection of antigen. Levels of significance (P) were computed on the $\log_{10}$ value of the stimulation indices by the analysis of variance procedure. * = $P < 0.05$. ** = $P < 0.01$.

	Days post-infection	25 g CR	50 g CR	25 g SR
Controls	0	0.9 ± 0.13	1.0 ± 0.2	1.1 ± 0.11
n =		55	55	55
Viable P-2383	1-14	1.3 ± 0.36	1.1 ± 0.21	2.7 ± 1.42
	n = 8			
	15-20	0.9 ± 0.07	1.1 ± 0.20	1.0 ± 0.14
	n = 8			
	20	1.2 ± 0.21	1.3 ± 0.18	1.0 ± 0.15
	n = 9			
P-2383 crude ribosomes	1-14	0.9 ± 0.13	0.9 ± 0.12	1.2 ± 0.21
	n = 7			
	15-20	0.6 ± 0.24	0.6 ± 0.07	0.8 ± 0.09
	n = 7			
	20	1.4 ± 0.30	1.6 ± 0.37	2.7 ± 1.30
	n = 7			
P-2383 RNA-protein	1-14	0.7 ± 0.15	1.0 ± 0.19	0.8 ± 0.12
	n = 7			
	15-20	0.8 ± 0.18	0.70 ± 0.13	1.0 ± 0.15
	n = 6			
	20	1.8 ± 0.33**	1.9 ± 0.36**	2.3 ± 0.66

50 g SR	25 g RNA-protein	50 g RNA-protein
0.8 ± 0.08	1.7 ± 0.66	0.8 ± 0.07
55	55	55
1.4 ± 0.62	2.3 ± 0.78*	1.7 ± 0.3*
0.9 ± 0.14	1.3 ± 0.17	1.2 ± 0.17
0.9 ± 0.11	2.0 ± 0.64	1.2 ± 0.19
0.8 ± 0.15	1.1 ± 0.16	1.0 ± 0.10
0.7 ± 0.14	2.2 ± 1.21	0.8 ± 0.13
1.8 ± 0.67	1.9 ± 0.83	3.0 ± 1.44**
0.7 ± 0.10	1.1 ± 0.14	0.8 ± 0.15
0.9 ± 0.15	0.9 ± 0.12	0.9 ± 0.17
2.4 ± 0.82**	4.2 ± 1.84**	3.3 ± 1.76**

control data consist of all observations on Group 2 cattle prior to antigen injection. Levels of significance (P) were computed on the $\log_{10}$ value of the stimulation indices by the analysis of variance procedure.

P-2383 RNA-protein stimulated a significant ($P < 0.05$) blastogenic response in cattle injected intradermally with viable P-2383 during the 1-14 day post-injection period. Mean stimulation indices of 2.3 $\pm$ 0.78 and 1.7 $\pm$ 0.30 were observed for the 25 and 50 µg levels of RNA-protein. The SR antigen produced a mean stimulation index of 2.7 $\pm$ 1.4 at the 25 µg level but this was not statistically significant. Significant stimulation of sensitized peripheral blood lymphocytes was not observed with the 50 µg level of SR or the 25 and 50 µg levels of the CR antigen at any time period.

P-2383 RNA-protein antigen stimulated a highly significant ($P < 0.01$) blastogenic response in cattle injected intradermally with the CR antigen during the > 20 day time period. The mean stimulation index was 3.0 $\pm$ 1.44. The mean stimulation index for the SR antigen during the same time period was 2.7 $\pm$ 1.30 at the 25 µg level. This did not reach significance.

The CR antigen, RNA-protein antigen and SR antigen all stimulated bovine lymphocytes at highly significant ($P < 0.01$) levels in cattle injected with RNA-protein intradermally. All significant stimulation occurred in the > 20 day time period. The mean stimulation indices for the 25 and 50 µg levels of CR antigen were 1.8 $\pm$ 0.33 and 1.9 $\pm$ 0.36 respectively. The 25 µg level of SR antigen produced a mean stimulation

index of 2.3 ± 0.66 which was significant ($P < 0.05$). The 60 µg level of the same antigen produced a mean stimulation index of 2.4 ± 0.82 which was highly significant ($P < 0.01$).

The RNA protein antigen produced the highest mean stimulation indices of the three antigens presented in Table 10. Values of 4.2 ± 1.84 and 3.3 ± 1.76 were observed for lymphocyte cultures from calves injected intradermally with P-2383 RNA-protein antigen when either 25 or 50 µg of P-2383 RNA-protein antigen was added to each well of the multiwell plate. Both values were highly significant ($P < 0.01$).

DISCUSSION

Prior to commencing a discussion of the investigation described herein, it was believed advisable to provide those uninitiated to the various typing schemes a brief review of those schemes utilized in this discussion. This list is in no way complete and is an extreme over-simplification but hopefully, will contribute to the understanding of the discussion at hand. Carter's (1967a) typing scheme has been the most widely utilized in the literature and a majority of the discussion will utilize this scheme.

		Species Affected	Disease
Carter's			
Serotype	A	Cattle	Pneumonic
		Rabbits	Pneumonic
		Avian	Fowl Cholera
	B & E	Cattle and Buffalo	Hemorrhagic Septicemia
	D	Porcine	Pneumonic and Turbinate Infections
Robert's			
Serotype	I	Cattle and Buffalo	Hemorrhagic Septicemia
	II	Encompasses elements of Carter's Types A & D but are not well correlated (Brogden et al., 1977).	
	III		
Heddleston's			
Serotypes	1-16	Pertains only to avian Pasteurella multocida and thus subdivides Carter's Type A avian isolates.	

The goal of this investigation was to elucidate the mechanisms involved in the immune responses of cattle to Serotype A Pasteurella multocida. Until very recently, definitive investigations of immunity to P. multocida have involved serotypes other than Type A or species other than the bovine. A comparative dearth of information exists in this area as a result. Both humoral and cell mediated responses have been postulated as the mechanism of resistance of cattle to P. multocida (Carter 1967b, Collins 1977, Maheswaran and Thies 1979). The humoral response in other species has been well documented by other workers (Carter, 1967b), while the humoral response of the bovine has been demonstrated but not extensively studied (Carter 1955, Heddleston et al., 1962). An attempt has been made to evaluate both the humoral and cellular responses of cattle to this bacterium using more definitive immunological and immunochemical methods which have become available in recent years.

Investigation of the humoral response to the antigens of P. multocida hinges upon the ability to produce relatively pure antigenic materials and yet maintain antigenicity. Several methods of antigen extraction were employed with varying results. Phenol-water extraction of P. multocida P-2383 yielded a fraction which gave two lines of precipitation in a gel diffusion precipitin test. This is consistent with the two precipitin lines observed by MacLennan and Rondle (1957) and Penn and Nagy (1974) for Types B and E P. multocida. The two antigens of P-2383 phenol-water extracts were very similar in their diffusion properties and on most occasions the lines tended to be

indistinguishable from one another.

The antigens associated with the phenol-water extracts were also observed in many of the other antigen preparations. At least one of them was present in the heat-extracted capsular materials from P-2383 and P-1062, the CR antigen, and the RNA protein antigen. No endotoxin components were observed in the SR antigen.

It is generally recognized that lipopolysaccharides of gram negative bacteria produce a nonspecific blastogenic response in lymphocyte cultures (Oppenheim and Perry, 1965). However, reports indicate some species differences in this response. Mouse lymphocytes respond nonspecifically with an almost entirely B cell stimulation; however, human lymphocytes do not respond well to lipopolysaccharides. In human lymphocyte cultures, lipopolysaccharides stimulate only B cells of sensitized subjects and do so only in the presence of some T cells; it, therefore, behaves like a T-dependent B cell antigen (Oppenheim and Perry, 1965; Ivanyi and Lehner, 1975). In this investigation, phenol-water-extracted lipopolysaccharide of P-2383 was not observed to generate a significant blastogenic response in bovine lymphocyte cultures. Thus, this lack of reactivity probably represents a species difference similar to that observed in man. Also, a specific blastogenic response of presumably sensitized bovine lymphocytes could not be demonstrated. That this is probably a characteristic of the bovine lymphocyte and not of the lipopolysaccharide itself is indicated by the nonspecific response to the same lipopolysaccharide in mouse lymphocyte cultures (Unpublished data). Thus it appears that

cattle may not respond to endotoxin with either a specific or non-specific B cell stimulation. It must be cautioned, however, that the lack of reactivity observed in bovine lymphocyte cultures towards the lipopolysaccharide moiety of P-2383 may reflect the relatively degraded nature of this antigen. Heddleston and Rebers (1975) observed that phenol-water extracts of P. multocida were incapable of inducing active immunity in mice, chicks and rabbits. Their investigations demonstrated that a protein moiety found associated with their free endotoxin was essential for immunogenicity. The absence of this protein moiety in the lipopolysaccharide of P. multocida may also affect the blastogenic response observed in the bovine. Therefore, any concrete conclusion on the reactivity of lipopolysaccharide of P. multocida in the bovine immune system must take into account the presence of a protein moiety which may alter this reactivity.

Free endotoxin from P-2383 was not extensively studied in the investigation. As would be expected from an endotoxin type material, it demonstrated a nonspecific potentiation of blastogenesis in bovine lymphocyte cultures. This effect was quite erratic and of a low order. Stimulation indices for control calves were observed to range from below 1.0 up to 5 or 6. The particular preparation utilized was not useful in indirect hemagglutination. Preparation of this material by ultrafiltration of whole broth cultures probably ensured the inclusion of high molecular weight media constituents. Thus, any blastogenic response could not have been directly attributed to the

free endotoxin present in this material. The presence of this low order response, however, does lend credence to the hypothesis that bovine lymphocytes may behave similar to human lymphocytes when relatively undenatured endotoxin preparations are utilized. A protein moiety associated with the lipopolysaccharides of P. multocida may, therefore, be essential to stimulate blastogenesis of bovine lymphocytes.

Maheswaran et al., (1975) demonstrated a specific blastogenic response in cultures of turkey lymphocytes with a free endotoxin preparation from agar grown P-1059. Maximum stimulation, observed at 4 µg/culture, gave the poorest blastogenic response of the antigens which they investigated. The presence of a specific blastogenic response in sensitized turkey lymphocytes serves to point out the marked species differences in reactivity which may occur.

It must be pointed out that the requirement of a relatively pure antigenic entity is probably not met by free endotoxin. This material is of a particulate nature (Ganfield et al., 1976). It is apparently composed of relatively undenatured cell wall material and, therefore, would be expected to contain some capsular polysaccharide and possibly other antigenic moieties. Thus, it would appear premature to ascribe a specific blastogenic response to the lipopolysaccharide-protein entity of P. multocida.

The use of TCA-extracted endotoxin offered no advantages over the phenol-water extracted material. The difficulty in obtaining this fraction in quantities sufficient for investigation limited its

applicability in serologic tests. Extensive use of this preparation in lymphocyte blastogenesis investigations proved disappointing. At no time was a dramatic response observed with this antigen. It therefore appears that weak TCA extraction of P-2383 either 1) denatures a component of the bacterium responsible for a blastogenic response or 2) does not extract such a component from the cell walls of P-2383. Which of these two situations is occurring is not known at this time and is worthy of further investigation.

The saline extracted capsular antigen was observed to be only slightly reactive in indirect hemagglutination and nonreactive in gel diffusion precipitation and immunoelectrophoresis. It did, however, elicit a specific blastogenic response in bovine lymphocyte cultures, albeit to a low degree. A mean stimulation index of 2.3 ± 0.9 was observed between days 1-14 following injection of viable P-2383. In addition, a response deemed to be significant by analysis of covariance but of very low order was observed in cattle which received the P-2383 RNA protein preparation intradermally. This response was observed late (> 20 days) in the post-injection period. A specific blastogenic response, therefore, is present in lymphocyte cultures of cattle sensitized with either the viable P-2383 or the RNA-protein antigen of this bacterium. Again, it must be emphasized that the nature of the antigen or antigens giving rise to this response is unknown since the saline extract contains both endotoxin and polysaccharide moieties and possibly other bacterial components as indicated by Penn and Nagy (1974).

It is interesting to note that the CR preparation injected intradermally into cattle did not sensitize lymphocytes to the saline-extracted antigen. The only easily detected difference between the CR and the RNA-protein preparations was the presence of the E antigen as observed in the immunoelectrophoretograms. However, it would be extremely premature to assign the role of a specific sensitizing antigen to this entity. There may be other qualitative as well as quantitative differences not apparent in these two preparations.

The "capsular" preparations of P-2383 and P-1062 obtained by heat-extraction were readily demonstrated to contain antigenic materials by the IHA test. Analysis of these materials by gel diffusion precipitation and immunoelectrophoresis, however, proved difficult. Both materials probably contain endotoxin and capsular polysaccharides. Weak precipitation lines were observed on gel diffusion precipitation which corresponded to the position of the endotoxin lines present in the phenol-water extracts. The capsular extracts appeared to share at least one other component with the CR, SR and RNA-protein antigens. This may correspond to capsular polysaccharides which theoretically should be present in most of these fractions. A lymphocyte blastogenic response was not generated by most of these fractions. Lymphocyte blastogenesis was not demonstrated with the heat-extracted capsule of P-2383. This is contrary to the findings of Maheswaran, et al., (1975) with turkey lymphocytes. Their investigation found a highly specific blastogenic response to this type of antigen. Differing results between these two investigations may indicate: 1) a difference between the antigenic makeup of the Pasteurella strains involved or 2) differences

between the responses of turkeys and cattle to this component of P. multocida.

A major problem observed in this investigation was the toxicity associated with the heat-extracted capsular preparation. The capsular material was demonstrated to be more toxic to bovine peripheral blood lymphocytes than any other fraction studied. At higher concentrations, stimulation indices below 1.0 and often less than 0.5 were observed. The toxicity of such capsular extracts is interesting to note in light of the demonstrated toxic effects exhibited by Pasteurella hemolytica on alveolar macrophages (Markham and Wilkie (1980). Heat-extracted capsular antigen of P-2383 was demonstrated to markedly inhibit the iodination reaction of bovine polymorphonuclear leukocytes in vitro (Unpublished data). The relationship of the demonstrated toxicity of this preparation to its importance as a protective immunogen warrants clarification.

Crude polysaccharide prepared from spent culture media of P-2383 demonstrated a low order of reactivity in all assays for antibody. Miller et al., (1975) observed that their crude polysaccharide antigen isolated from Hemophilus somnus also demonstrated little serologic reactivity in cattle. This fraction received extensive chemical and physical treatment. One hypothesis is that such treatment may have altered the chemical or physical makeup of this material resulting in lowered antigenicity. Another hypothesis is that cattle may not form significant amounts of antibody to the polysaccharides of P. multocida. Bush and Kaeberle (Unpublished data) have demonstrated

that such a situation exists with Hemophilus somnus in cattle. Rabbits were observed to readily produce demonstrable antibody to a crude polysaccharide material prepared similarly to the one utilized in this work. Cattle, however, produced no demonstrable antibody to the same crude polysaccharide. Schutze (1932) demonstrated that different components of Yersinia pestis were responsible for immunity in different animal hosts. Thus, direct extrapolation of data obtained in species other than cattle may not be entirely valid.

Investigations in which certain properties have been ascribed to the capsular polysaccharides of P. multocida have been misleading. To this author's knowledge, no purified polysaccharide component of P. multocida yielding only a single precipitin line on both gel diffusion-precipitation and immunoelectrophoresis has yet been investigated in cattle.

Historically, the role of protective immunogen has been ascribed to either the capsular polysaccharide or the lipopolysaccharide moiety of P. multocida. Indeed, one, or both, may very well be the protective immunogen in septicemic types of pasteurellosis. However, Mukkur and Nilakantan (1969) and Alexander and Soltys (1973) have indicated that such a relationship between protection and the levels of antibody fractions may not exist in hemorrhagic septicemia and fowl cholera. It was observed that the antibody titers against these fractions obtained in their investigations had a negative correlation with protection. Animals with higher antibody titers were observed to have less protection against challenge with P. multocida than animals with lower

titers. The majority of investigations, however, indicate that immunity to P. multocida is humoral in nature in septicemic pasteurellosis. The exact nature of this immune response and knowledge of which antigens are protective remains unclear at present.

The nature of an effective immune response of the bovine to pneumonic pasteurellosis may represent a completely different situation from that observed in septicemic types of pasteurellosis. Such considerations are of importance in light of the possible toxicity of the capsular polysaccharide and lipopolysaccharide antigens. Such a situation is indicated by the toxicity associated with the heat-extracted capsule of P-2383 in cultures of bovine lymphocytes. In addition, large amounts of capsular polysaccharides may interfere with the immunological response of the bovine to other antigenic materials. Nakashima et al. (1971) demonstrated a decreased serum antibody response in mice to bovine serum albumin (BSA) when capsular polysaccharides of Klebsiella pneumoniae were injected 3 to 30 days prior to injection of BSA. In cattle, stress has been associated with so-called "vaccine breaks" in which recently vaccinated animals are affected with pneumonic pasteurellosis. Such stress factors may include a nonspecific immunosuppression caused by the capsular or cell wall components of commercial bacterins. Definitive proof for such a theory has yet to be demonstrated.

Crude polysaccharide from P-2383 was also lacking in ability to stimulate a blastogenic response in cultures of bovine lymphocytes. This material was not included in later work for this reason. This

fraction possessed toxicity similar in nature to that observed with the heat-extracted capsular preparation of P-2383 in lymphocyte cultures.

The CR, SR and RNA-protein fractions of P-2383 presented the most complex antigenic makeup of the bacterial fractions utilized in this investigation as observed in gel diffusion precipitation and immuno-electrophoresis. Whether this distinction is qualitative or quantitative in nature is difficult to assess in light of the relatively poor and ill-defined reactions observed with the other antigens employed in these tests. Of all the antigens studied, the CR, SR and RNA-protein preparation, were subjected to the least chemical and physical degradation. This in all probability preserved the antigenicity observed with these materials and facilitated their study.

The CR and RNA-protein preparations were clearly observed to contain lipopolysaccharide-like materials by gel diffusion precipitation. The methods by which these fractions were prepared ensured the inclusion of endotoxin. The ultracentrifugation of the CR antigen at 108,000 g is similar to accepted procedures for preparation of endotoxin as is the ethanol precipitation employed in production of the RNA-protein antigen (Staub, 1967). Attempts to reduce the amount of endotoxin present in these preparations by centrifugation of the disrupted bacteria at 65,000 g for 1 hr. were only partially successful. The SR preparation did not contain any demonstrable endotoxin as observed in gel diffusion precipitation.

The CR, SR and RNA protein fractions were observed to contain

components on gel diffusion precipitation which formed lines of identity with the heat-extracted capsular materials of P-2383 and P-1062, although the exact relationship could not be clearly defined. The use of immunoelectrophoresis was of no assistance in elucidating the relationship of these antigens. Presumably the CR, SR and RNA-protein fractions, should contain capsular constituents since no effort was made to exclude these materials. The relatively sharp, well-defined antigenic makeup of the CR, SR, and RNA-protein fractions compared with the heat-extracted capsules may result from the inclusion of interfering materials in the latter. Carter (1967b) has indicated that the capsule of Type A P. multocida contains large amounts of hyaluronic acid. This or possibly other constituents may be released more readily upon heating of the bacteria. Such nonantigenic moieties may interfere with either: 1) the diffusion of the antigens in the heat extracts leading to a blurring effect or 2) the precipitation reaction between the antigens and antibodies in the system. The difference in these antigens may also result from a low concentration of specific antigens in the heat extracts. Concentration of these fractions by removal of water resulted in an increasingly viscous material which was of no use in either gel diffusion precipitation or immunoelectrophoresis. That a higher concentration of antigens in the SR fraction was responsible for the clear pattern of precipitin lines is a hypothesis not supported by the indirect hemagglutination results. Although such relationships are not entirely quantitative, it required a larger volume of the SR fraction to sensitize SRBC than it did of the heat-extracted capsular

preparations. Thus, either more of the specific antigen(s) were present in the latter or it was present in a state which was more readily coupled to SRBC. Lastly an obvious explanation of the differences between these fractions may be that the antigens are not the same. As observed in the gel diffusion precipitation tests, there is no conclusive demonstration of identity. A more definitive comparison awaits the clarification of the antigenic makeup of the heat-extracted capsular materials.

Differences were observed between the antigenic makeup of the CR, SR and RNA-protein antigens of P-2383 on gel diffusion precipitation and immunoelectrophoresis. By immunoelectrophoresis, antigen D was observed to be present in the SR and RNA-protein preparations and absent from the CR preparation. Thus, it appears that the D antigen is of relatively low molecular weight since it was not present in the CR pellet obtained on ultracentrifugation.

Other marked differences were demonstrated between these fractions. Overall, the SR preparation contained only three antigens compared with at least 5 and possibly more associated with the CR and RNA-protein preparations. The SR preparation lacked antigen A as observed in immunoelectrophoresis. The absence of antigen A in the SR preparation and its presence in the ultracentrifuged CR preparation indicates that this antigen has a relatively high molecular weight. The great mobility of this antigen when electrophoresed indicates the presence of a large number of negatively charged groups. Both conditions are consistent with a large molecular weight polysaccharide moiety. It is

of interest to note that, in the absence of antigen A, a highly significant blastogenic response was generated in lymphocyte cultures of cattle injected intradermally with the RNA-protein preparation. Stimulation indices of 2.3 ± 0.66 and 2.4 ± 0.22 were observed for the 25 µg levels of SR antigen in cattle immunized intradermally with the RNA-protein preparation. This compares with values of 4.2 ± 1.84 and 3.3 ± 1.76 for the RNA-protein preparation at the same dosage rates respectively and in the same cattle during the same time period. The CR fraction which contained the A antigen gave stimulation indices of 1.8 ± 0.33 and 1.9 ± 0.33 respectively for the 25 and 50 µg levels. Thus, this specific blastogenic response must be generated by a component other than the A antigen. This narrows the choice down to the B, C, and D antigens as observed in the immunoelectrophoretograms. However, it must be cautioned that more than one of these antigens may be involved in such a response. It is also of great interest to note that a specific blastogenic response occurred in the absence of the high molecular weight, endotoxin-like material observed in the RNA-protein preparation. It appears, therefore, that the entire blastogenic response cannot be attributed to a T cell dependent, B cell blastogenesis caused by lipopolysaccharides similar to that described in cultures of human lymphocytes (Oppenheim and Perry, 1965).

The SR preparation contained the B and C antigens as indicated by the immunoelectrophoretograms. Prince and Smith (1966a, b) observed an antigen which moved electrophoretically very similar to

either of these two antigens. They labeled this the β antigen and demonstrated its existence in all Pasteurella multocida as a heat stable, type specific antigen. Their β antigen was demonstrated to be polysaccharide in nature. The resemblance between the B and C antigens of P-2383 and the β antigen of Prince and Smith is, at best, superficial. Further investigation must be carried out to define the relationship between these antigens.

The presence of antigens B and C in the immunoelectrophoretograms of all three fractions was of great interest. Their presence in both the supernatant and pellet from the disrupted bacteria indicated that these antigens existed in both low and high molecular weight forms. Two explanations are readily apparent: 1) The antigens involved may have been capsular polysaccharide or other cell wall constituents which varied in their degree of polymerization and thus their molecular weight. 2) The antigens may have been of uniform size and molecular weight but a portion of them were found free in the supernatant and the rest were bound to other cellular constituents. Such constituents could have been cell wall fragments or cellular organelles. The possibility exists that a portion of the B and C antigens could have been bound to the ribosomes of P-2383 and another portion of B and C not bound. If this was true, then the B and C antigens are protein in nature and represent proteins caught in the act of being synthesized on the ribosomes. However, the work of Prince and Smith (1966a, b) would indicate that at least one of these is polysaccharide in nature.

The B and C antigens were highly mobile on immunoelectrophoresis indicating the presence of a large number of acidic groups. This is consistent with a polysaccharide entity. B and C could also have a protein associated with them. Such a protein could form a portion of the cell wall of P. multocida. Rebers and Heddleston (1974) indicate that a protein moiety is essential for immunogenicity of free endotoxin which arises from the cell walls of P. multocida. The presence of the B and C antigens in both the CR and SR fractions indicates that they must be present in an unbound form as well as a constituent of or in association with other components. It appears that the most likely explanation is that B and C are cell wall constituents.

The D antigen was an antigen observed in the immunoelectrophoretograms of the SR and RNA-protein preparations but not the CR preparation. Thus, this may be an antigen involved in the superior blastogenic response seen with the SR and RNA-protein antigens in bovine lymphocyte cultures. The electrophoretic mobility of this antigen indicates it to be low in charged groups. Precise definition of this antigenic constituent must await further investigation.

Another readily apparent distinction between the CR, SR, and RNA-protein preparations was the presence of the E antigen in the latter as observed in the immunoelectrophoretograms. The presence of this antigen only in the RNA-protein fraction may have some bearing on the superior blastogenic response observed in bovine lymphocyte cultures in this investigation. Its exact role cannot be ascertained from the

observed data and elucidation of its role must await purification and characterization of this antigen. It can be noted from the immunoelectrophoretograms that antigen E did not move readily when electrophoresed. Indeed it appeared to move in both directions from the antigen well. It was, therefore, composed of molecular constituents which have an isoelectric point near pH 8.2. It was observed to diffuse readily through the agar indicating a molecular weight similar to that of the D antigen. The indications are that this is a protein antigen.

One disturbing point must be brought out concerning the E antigen. Theoretically, it should be present in either the CR or SR fractions. This is because the RNA-protein fraction has, as its starting material, the raw material for the CR and SR fractions; nothing is discarded and yet an additional antigen appears when starting material is precipitated with ethanol rather than ultracentrifuged. The only possible explanation is that this fraction may become aggregated with another component of the CR preparation during ultracentrifugation. Thus, it is not observed in the immunoelectrophoretograms of the CR fraction. It is germaine to note here that minor variations did exist in the positions of the other component antigens in the CR fractions when compared to the SR and RNA-protein fractions. Also an H antigen appeared in the CR preparation which was not present in either the SR or RNA-protein fractions. It is conceivable that this antigen may be involved in such an aggregation effect. The aggregation of the E

antigen with another component could have conceivably altered its immunogenic and blastogenic capabilities. Thus, another intriguing question is presented which is worthy of further investigation.

Another interesting antigen was the G antigen observed in immunoelectrophoresis of all preparations except the RNA-protein and phenol-extracted endotoxins. This antigen appears to be identical to the α antigen present in the immunoelectrophoretograms of Prince and Smith (1966a, b). These investigators determined that this antigen was not highly heat stable. The relative weakness of this precipitin line in the immunoelectrophoretograms of the heat-extracted capsules of P-2383 and P-1062 is readily explained in light of this fact. The nature of this component is difficult to ascertain. It moves extremely heterogeneously on electrophoresis. Thus, there must be a marked variation in the number of charged groups on its constituent antigens. It does not diffuse readily through the agar as demonstrated by its formation of a line midway between the antiserum troughs. Thus, it may be assumed to be of a relatively high molecular weight. One could reasonably expect a capsular or cell wall polysaccharide bound to a variable amount of protein to behave in this fashion. Prince and Smith (1966a, b) determined that this antigen was susceptible to proteolytic digestion. The heat lability and the susceptibility to proteolytic digestion of this antigen may be a function of an accompanying protein moiety. Again, this is merely speculation and definitive proof awaits further investigation. It can be stated, however, that this antigen probably played no role

in the specific lymphocyte blastogenesis which was observed in this investigation. Indeed, this antigen may be responsible for some or all of the toxic effects observed with the various fractions. The superior blastogenic response of bovine lymphocytes stimulated with the RNA-protein preparation which apparently lacks the G antigen could possibly be a result of the absence of this antigen. Thus, there may have been a decrease in the amount of lymphocytotoxic material present in the RNA-protein fraction.

The employment of the SR antigen in the indirect hemagglutination test was of considerable value. It appeared to be the most sensitive test of recent antigenic stimulation by P. multocida. Thus, it was of use to screen animals for prior exposure. On gel diffusion precipitation and immunoelectrophoresis the RNA-protein preparation was observed to contain the same three antigens found in the SR preparation. Thus, it must be assumed that the use of this fraction in the indirect hemagglutination test should be successful. The CR preparations should also be useful in this test. The major obstacle to their use was their hemolyzing effect on sheep red cells. If this problem with hemolysis could be overcome, both the CR and RNA-protein fractions should be able to specifically sensitize SRBC. The SR preparation also was capable of causing enough hemolysis to interfere with the coupling of this antigen to SRBC by glutaraldehyde. This effect was minimized by carefully controlling the temperature of the reaction mixture. At temperatures above 25° C the hemolysis occurred readily, although to a low degree. The hemolysis superficially appears to be

an enzyme mediated phenomenon. Gel electrophoresis has demonstrated the presence of multiple protein bands in the SR fraction, some of which may represent bacterial enzymes. It is, therefore, not unexpected that one or more of these may act upon the red cell membrane to produce a lytic effect.

It is also interesting to note that significant levels of antibody were generated in cattle by intradermal injection of the CR and RNA-protein preparations. Analysis of covariance revealed that no difference existed between antibody levels generated by these two preparations and the antibody level generated by intradermal injection of viable P-2383. Two important facts are thus brought out: 1) the intradermal injection of cattle does generate a significant antibody response and 2) no differences were observed in the degree of this response between those cattle receiving viable P. multocida or those receiving the CR or RNA-protein preparations. On the basis of the limited results obtained in this investigation, it would appear that intradermal injection of cattle is capable of producing a good immune response. With the present methods, large scale intradermal injections of cattle would be laborious and time consuming. If a method for rapid intradermal injections were available, such a route of inoculation could be very useful. At the present time, however, intradermal injections of cattle on a large scale are not feasible.

The use of the word "ribosome" may be a misnomer for the CR preparation. This fraction was observed to contain many antigenic materials, including endotoxin-like antigens and capsular antigens.

In the absence of gel diffusion precipitation or immunoelectrophoretic analyses of the ribosome preparation of Baba (1977) it is impossible to compare the CR fraction with his material. It is of great interest to note that immunogenicity of such ribosomal vaccines have been attributed to the presence of ribosomal RNA. Youmans and Youmans (1973), found that the protective activity of RNA from Mycobacteria lies not in the single-stranded but in the double-stranded 23S RNA. However, Baba (1977) found no protective effect at all in RNA from P. multocida. A protein moiety is apparently essential for full immunogenicity. The RNA in the case of P. multocida is probably acting as an adjuvant.

It is becoming increasingly apparent that the immune response to P. multocida is not as straightforward as it is for many other bacterial agents. In 1977, Collins reviewed the data available at that time on the mechanism of the immune response to P. multocida. He concluded that all evidence indicated the immune response was totally humoral in nature. He based this conclusion on some excellent studies of the growth of P. multocida in both immune and nonimmune mice. However, his work raised some disturbing questions on the nature of this humoral immunity.

Collins (1973) observed that in nonimmune mice, 98% of a peritoneal challenge remained in an extracellular phase in peritoneal washout cultures. The addition of immune serum prior to peritoneal challenge resulted in 95% of the bacterial population becoming cell associated. However, in the latter case, the rate of inactivation of

the phagocytosed organisms was not increased. In fact, viable counts were observed to increase. The addition of penicillin was unable to halt this increase in viable counts. Even macrophages taken from the peritoneal cavities of demonstrably immune donors were unable to halt the growth of viable bacteria. Thus, it appears that P. multocida is capable of multiplying within peritoneal macrophages regardless of the presence of specific opsonins and/or cells from immune mice. Experimentation in our laboratory on the killing of P. multocida by bovine polymorphonuclear leukocytes (PMNs) in vitro indicated a similar resistance to phagocytosis and killing (Unpublished data). In the presence of immune serum and PMNs, fully virulent P-2383 were observed to increase in numbers over cultures containing only immune serum and bacteria. Multiplication of a blue dissociant of P-2383 in the presence of PMNs and immune serum was observed to be significantly reduced when compared to cultures of P-2383 and immune serum. By dissociating from a fully virulent, iridescent form to a relatively nonpathogenic, blue form, P-2383 lost the ability to resist phagocytosis and killing.

The implications of the above findings are disturbing in light of the demonstrated humoral immune response to P. multocida. Woolcock and Collins (1976) indicated that, even in fully protected mice, dramatic growth of P. multocida occurred in the livers and spleens of intravenously challenged mice. The numbers of viable bacteria recovered from each organ increased 10 to 50 fold over the number of challenge organisms injected intravenously into a given mouse. Such growth

occurred for up to seven hours in mice vaccinated subcutaneously with two doses of heat-killed P. multocida incorporated into either Freund's complete or incomplete adjuvant. Mice vaccinated 16 days prior to challenge with live BCG followed by 200 µg heat-killed P. multocida 4 days prior to challenge demonstrated similar in vivo growth of P. multocida up to 18 hours following challenge. These findings indicate that P. multocida is able to resist to some extent the normal mechanisms utilized by phagocytic cells to kill ingested bacteria. It was concluded that immunity to P. multocida is humorally mediated; however, there appear to be factors involved which have not been explained. Heddleston and Watko (1965) and Bhasin and Biberstein (1968) concluded that neither the agglutination nor passive hemagglutination reactions gave a true indication of immune status in vaccinated birds subsequently exposed to virulent P. multocida. Alexander and Soltys (1973), observed a negative correlation in turkeys in some cases between serum agglutination titers and immunity to challenge. Serum with a titer of 640 was found to be less protective than serum with a titer of 160. In all instances, immunity to challenge was greater at 35 days post immunization when antibody titers had fallen off considerably than it was at 19 days post immunization when agglutinin titers were at their peak.

Two conclusions can be drawn from the above discussion: 1) immune serum alone is not bactericidal to fully virulent serotype A P. multocida and 2) immune serum does not significantly increase the capacity of polymorphonuclear leukocytes to phagocytose and inactivate

fully virulent serotype A P. multocida. In light of the observations of Collins (1977) on the in vivo growth curves of P. multocida the the nature of this humoral protection remains an enigma. The possibility exists that immunogens other than those reacting in the agglutination and passive hamagglutination tests are important in the immune response. P. multocida presents a very complex antigenic mosaic as indicated by the gel diffusion and immunoelectrophoresis results presented in this investigation. Prince and Smith (1966a, b) and Baxi et al. (1970) also present evidence for the complexity of the antigenic makeup of P. multocida. Many workers have concluded that the protective immunogen is capsular in nature and others that the protective immunogen is the endotoxin moiety. The above results indicate that perhaps neither may be responsible for protective immunity in the species named.

The results presented in this investigation have demonstrated the presence of a specific blastogenic response in bovine lymphocyte cultures to certain antigenic fractions of P. multocida. Cattle immunized intradermally with viable P-2383, CR and RNA-protein fractions were observed to have increased stimulation indices particularly with CR and RNA-protein antigens. The presence of a lymphocyte blastogenic response in cattle indicates that the mechanism of immunity to P. multocida, although long considered to be entirely humoral in nature, may also involve a cellular response. The results presented here concur with those published by Maheswaran and Thies (1979). Using whole blood cultures, these workers were able to

demonstrate a highly specific response in immunized cattle to a sonicated soluble fraction (SSF) of P. multocida. This fraction bears a superficial resemblance to the starting material for the CR, SR and RNA-protein antigens utilized in this investigation. The SSF was prepared by sonication of viable P. multocida followed by centrifugation to remove particulate debris. Such preparations undoubtedly preserve antigenicity to a greater extent than can be maintained with other extraction procedures. It would have been of significant interest if the authors had related blastogenic responses with serological responses in their cattle. It is also interesting to note that they produced specific immunosensitization through the administration of a formalin-killed bacterin. In our preliminary studies, cattle immunized with heat-killed bacterins incorporated in aluminum hydroxide gel, did not demonstrate a blastogenic response to P. multocida antigens. It must be pointed out that the dosage rate of bacterin used in our preliminary work was much lower than that employed by Maheswaran and Thies.

The presence of a possible cell-mediated response to certain antigenic fractions of P. multocida is of importance in understanding the entire mechanism of the immunity or susceptibility of cattle to P. multocida. Cell mediated responses have been demonstrated for such bacterial agents as Mycobacteria, Listeria, Brucella and Salmonella (Collins, 1974). Tizard and Ellicott (1974) were able to demonstrate macrophage migration inhibition reactions to Pasteurella hemolytica in mice. These authors were unable to demonstrate a delayed-type

hypersensitivity to either lipopolysaccharide or lipoprotein antigens. They did, however, elicit an Arthus reaction to these fractions. The macrophage migration-inhibition is in contrast to the findings of Collins and Mackaness (1968) who indicated that, for Salmonellae, lipopolysaccharide-containing cell wall antigens were not directly involved in cell-mediated responses.

Cell mediated responses to such bacterial agents as Mycobacteria, Salmonella and Listeria are much more apparent than those observed with P. multocida. Challenge of immune hosts with these agents leads to a predominantly mononuclear phagocytic cell response with little evidence of a polymorphonuclear infiltration of lesions. Collins (1977) observed that the response of immune mice to P. multocida was almost entirely polymorphonuclear in nature. Thus, with a systemic challenge, an immune response involving mediation by specifically sensitized cells of the mononuclear series was not observed.

The significance of the demonstrated lymphocyte blastogenic response lies in the fact that such a response could be demonstrated at all. Little evidence exists for a specific cellularly mediated mechanism playing an important role in systemic infections by P. multocida. However, in the immune response to aerogenic challenge such a mechanism could play a significant role. In view of the data available at this time, it seems somewhat naive to assume an entirely humoral mediation of resistance. The historic dichotomy of the immune system has given way in recent years to a realization that both cellular and humoral responses occur to many bacterial agents.

With Listeria and Salmonella infections in mice, both aspects of the immune system are called into play. Ultimate responsibility for elimination of the bacterium from the reticuloendothelial organs, however, rests with the specifically activated macrophage. A similar situation involving cells other than macrophages may exist with P. multocida. It has been demonstrated that good protection can be obtained by transferring immune serum into normal mice and chicks (Collins, 1977). However, what is not indicated is the longevity of such passive protection. In our work with passively protected mice, the mice demonstrated good immunity up to 5 days following challenge (Unpublished data). However, subsequent to this, mortality was observed to increase dramatically. The work of Collins and Woolcock (1976) and Woolcock and Collins (1976) is noteworthy for its lack of bacterial counts on mice after 4-6 days post challenge. It would be of interest, therefore, if a rebound of viable bacterial counts were observed in the tissues of passively protected mice 7 or more days following challenge. If it occurs, such a rebound of viable bacterial counts could reflect a decrease in the quantity of circulating antibody in passively protected mice. This could be due to catabolism of the immunoglobulins. Where adequate long-term protection does occur, de-novo antibody synthesis coupled with a cellular response may be responsible for the ultimate clearance of the residual P. multocida from the reticuloendothelial organs.

The mechanism of immunity to pneumonic pasteurellosis in cattle may bear little resemblance to that associated with systemic infections.

in mice. Even in mice, circulating antibody, demonstrated to be fully protective against intravenous challenge, was observed to be much less effective against aerogenic challenge (Woolcock and Collins, 1976). In both actively and passively immunized mice, an aerogenic challenge of P. multocida was observed to multiply at an unrestricted rate for longer periods of time than was observed with intravenous or intraperitoneal challenge.

Collins (1977) observed that much higher numbers of viable P. multocida were required for an effective aerogenic challenge of mice versus an intravenous or intraperitoneal challenge. Thus, there appears to be a great difference in the degree of protection when "immune" animals are challenged by an aerogenic route versus other routes. The nature of these differences have not been adequately addressed to date. The primary mode of natural challenge is certainly not intraperitoneal or intravenous; it is aerogenic. While protection against the hemorrhagic septicemia and fowl cholera entities of P. multocida infections can be measured by such parenteral routes, the only true measure of protection against pneumonic pasteurellosis is via an aerogenic challenge. Once colonization of the bovine lung has occurred and the host pulmonary immune reactions have been initiated, the eventual outcome appears to rest with the pulmonary defenses and not the systemic clearing mechanisms. Therefore, the production and testing of Pasteurella multocida bacterins on the basis of systemic challenge systems would appear inappropriate and possibly misleading.

The difficulties of establishing a reliable system for aerogenic

challenge of cattle have been well-documented (Carter 1967b). Various stress factors have been found to be essential to a successful challenge with this organism. Stress factors such as prior or concomitant infection with another infectious agent, shipping, or injection of corticosteroids have all been employed successfully.

Heddleston et al. (1962) and Hetrick et al. (1963) were able to induce a febrile response and respiratory illness in calves receiving aerosolized P. multocida and myxovirus parainfluenza 3 (PI_3). Hetrick et al. (1963) demonstrated a synergistic infection when PI_3 was administered 2 days prior to, simultaneously, or 2 days following intratracheal inoculation of P. multocida. Other investigators have found it necessary to employ similar stressing agents to establish aerogenic challenge routes with the related agent, Pasteurella hemolytica. Collier (1968) was able to effectively challenge calves with P. hemolytica by an aerosol route 30 days subsequent to intratracheal inoculation of infectious bovine rhinotracheitis virus. High febrile reactions and marked clinical signs were observed in 4 of 4 calves following the P. hemolytica challenge. Gilmour et al. (1975) were able to effectively challenge lambs with an aerosol of P. hemolytica. It appears, therefore, that aerogenic challenge routes are feasible if one is willing to accept the complications incurred by inclusion of a stressing agent within the experimental model. The use of various stress factors and viral agents to assist in establishing an aerogenic challenge route in cattle is not without merit. Stress factors associated with shipping are known to play an important role in pneumonic pasteurellosis although the mechanisms

involved have not been elucidated (Carter, 1967a). In addition to IBR and PI_3 viruses, bovine viral diarrhea virus and adenoviruses have been implicated as predisposing or synergistic agents. It appears, therefore, that an experimental model including various stressing agents coupled with an aerogenic challenge of P. multocida is a far more accurate measure of the protective response of cattle to various immunizing agents when compared to systemic challenge of mice.

Attempts have been made to study the various aspects of the pulmonary immune defense mechanisms. Two of the most important factors in the resistance of a host to aerogenic bacterial infections are alveolar macrophages and mucociliary clearance. Lung clearance studies have been used by several workers to investigate respiratory defense mechanisms. Green and Kass (1965) found that Staphylococcus albus was cleared from from mouse lungs more rapidly than either S. aureus or Proteus mirabilis. Cold, as an environmental stress, was not found to affect clearance rates, but wetting and exposure to cold significantly reduced clearance. Sellers et al. (1961) and Kass et al. (1966) demonstrated a severely impaired clearance of S. aureus subsequent to influenza virus infection in mice.

Several workers have studied the clearance of gram negative bacteria from the lung. Green and Kass (1965) found that Pasteurella pneumotropica was cleared from mouse lungs at a slower rate and with more variability than was S. aureus. Jackson et al. (1967) demonstrated that Pseudomonas aeruginosa multiplied rapidly for the first hour and only then did clearance begin. This situation bears a resemblance to

that of Pasteurella multocida in murine lungs (Woolcock and Collins, 1976). P. multocida multiplies freely for several hours before onset of bacterial clearance.

Investigations such as those described above point out the marked variation in the ability of the mucociliary blanket and pulmonary alveolar macrophages to clear various bacterial agents. Of those agents studied, virulent gram negative rods appear to resist such clearance mechanisms. Such resistance to the normal pulmonary clearance mechanisms may involve specific cytotoxic or immunosuppressive actions by components of the bacterial agents involved. Markham and Wilkie (1980) demonstrated that P. hemolytica introduced into cultures of bovine alveolar macrophages caused the release of ^{51}CR. Culture supernatants of P. hemolytica were observed to depress phagocytosis in alveolar macrophages at low concentrations and exhibit cytotoxicity at high concentrations. Schwab (1975) reviewed the data available on the suppression of the immune response by various microorganisms. Immune suppression has been demonstrated for Group A Streptococci, Corynebacterium parvum, Mycoplasma, Pseudomonas aeruginosa, Bordetella pertussis, Klebsiella pneumoniae and Escherichia coli. Specific immune suppression by components of P. multocida would therefore not be unexpected under certain circumstances.

Friend et al. (1977) presented evidence that systemic immunization with P. hemolytica can result in a more severe pneumonia upon challenge exposure, either naturally or experimentally, than that observed in nonvaccinated cattle. The results of Markham and Wilkie (1980),

indicate that bovine pulmonary antibody possessing opsonizing activity for P. hemolytica, may lead to death or damage of the pulmonary alveolar macrophages following phagocytosis. Under such circumstances, specific opsonizing antibody, rather than being beneficial, may enhance the severity of the disease process rather than mediate protection. These observations may in part explain the marked growth of phagocytosed P. multocida in peritoneal macrophages demonstrated by Woolcock and Collins (1976). These investigators demonstrated a similar increase in viable bacteria within the lungs of immune mice. It would be of great interest if specific opsonins were enhancing phagocytosis of P. multocida in the alveolar macrophage without an accompanying activation of that macrophage. By such a mechanism, a detrimental effect would be exerted on the immune response of the host. This would explain in part the relatively poor protective immune response observed with formalinized bacterins in bovine pneumonic pasteurellosis. If this is indeed the case, alternate immunizing agents should be found or the practice of vaccination of cattle with formalinized bacterins re-examined.

SUMMARY

The objectives of this investigation were to: 1) isolate and characterize various immunogenic fractions of Type A Pasteurella multocida and 2) characterize the nature of the immune responses of the bovine to these immunogenic fractions. A clinical isolate (P-2383) and a strain commonly utilized for production of bacterins (P-1062) were fractionated by various means. These fractions were characterized chemically for protein, ribonucleic acid and carbohydrate content.

Serologic procedures to analyze the various fractions demonstrated the presence of one or more immunogens in each fraction. Several of the preparations contained multiple immunogens and many of the immunogens were present in more than one fraction. The exact relationship of many of the immunogenic moieties and their role in protective immunity remains to be established.

Lymphocyte blastogenesis was utilized to investigate the role of each of the preparations played in a possible cellular response. It was demonstrated that at least three of the preparations were able to stimulate a specific blastogenic response in cultures of bovine peripheral blood lymphocytes. These were the CR, SR and RNA-protein fractions of P-2383. The saline-extracted capsular antigens of P-2383 stimulated a low level blastogenic response.

The intradermal administration of viable P-2383, CR and RNA-protein fractions elicited significant humoral responses. In addition a specific sensitization of the peripheral blood lymphocytes of the cattle so immunized was demonstrated.

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